DEVELOPMENT OF DIAGNOSTIC PLATFORM FOR DETECTION OF BIOLOGICAL AGENTS AND TOXIC MICROALGAE USING ISOTHERMAL AMPLIFICATION.

Olena Mayboroda

Development of diagnostic platform for detection of biological agents and toxic microalgae using isothermal amplification

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Doctoral thesis

UNIVERSITAT ROVIRA i VIRGILI

Tarragona, 2017

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Doctoral thesis supervised by

Dr. Ioanis Katakis

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CERTIFY:

That the present study, entitled "*Development of diagnostic platform for detection of biological agents and toxic microalgae using isothermal amplification*", presented by Olena Mayboroda for the award of the degree of Doctor, has been carried out under my supervision at the Department of Chemical Engineering of Universitat Rovira i Virgili and that it fulfils all the requirements to be eligible for the International Doctorate Award.

Tarragona, September 4th, 2017

Dr. Ioanis Katakis

Dr. Ciara K. O'Sullivan

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List of abbreviations

Abbreviation	*Definition*
3SR	Self-sustained sequence replication
ALP	Alkaline phosphatase
ASSURED	Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Deliverable to end users
ATP	Adenosine triphosphate
CDC	Centre for Disease Control and Prevention
CFU	Colony- forming units
DAT	Direct agglutination test
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
dsDNA	Double stranded deoxyribonucleic acid
DT1	10-(3,5-Bis((6-mercaptohexyl)oxy)phenyl)-3,6,9-trioxadecanol
DVD	Digital versatile disc
EIS	Electrochemical impedance spectroscopy
ELISA	Enzyme-linked immunosorbent assay
ELONA	Enzyme-linked oligonucleotide assay
FISH	Fluorescent in situ hybridisation
GMO	Genetically modified organism
GOD	Glucose oxidase
GPES	General Purpose Electrochemical System
HDA	Helicase-dependent amplification
HGP	Human Genome Project
HIV	Human immunodeficiency virus

HRP	Horseradish peroxidase
ISFET	Ion-sensitive field-effect transistor
IUPAC	International Union of Pure and Applied Chemistry
LAMP	Loop-mediated isothermal amplification
LOD	Limit of detection
mer	From Greek meros, "part". The length of an oligonucleotide
mRNA	Messenger RNA
NAAT	Nucleic acid amplification test
NASBA	Nucleic acid sequence-based amplification
PBS	Phosphate-buffer saline
PCR	Polymerase chain reaction
PMMA	Poly (methyl methacrylate)
POC	Point-of-care
qPCR	Quantitative real-time polymerase chain reaction
RCA	Rolling circle amplification
RNA	Ribonucleic acid
RPA	Recombinase Polymerase Amplification
SA-HRP	Streptavidin labelled horseradish peroxidase
SAM	Self-assembled monolayer
SDA	Strand Displacement Amplification
SERS	Surface-enhanced Raman spectroscopy
SNP	Single nucleotide polymorphisms
SPR	Surface plasmon resonance
SP-RPA	Solid-phase RPA
SSB	Single strand binding proteins
ssDNA	Single stranded deoxyribonucleic acid

TBE	Tris/Borate/EDTA buffer
TMA	Transcription mediated amplification
TMB	3,3′,5, 5′-tetramethylbenzidine
UV	Ultraviolet
uvsX	Recombinase protein
uvsY	Recombinase loading factor
WHO	World Health Organisation

Summary

An easy, rapid and reliable detection of multiple biological warfare agents, pathogens, viruses and toxins is required in different situations of molecular diagnostics: from personalised medicine to bioterrorism defence. The main goal of this Doctoral thesis is to present alternative amplification and detection platform that combines the isothermal amplification technique with a solid-phase detection system in order to overcome some of the currently existing limitations in the field.

This work presents a simple, rapid and easy to integrate isothermal amplification protocol that can be used for analysis of genetic material and has good chances to be applied in point of care devices. Ultimately, the proved multiplexing ability of the system makes it a promising candidate to be integrated in devises for in situ testing due to its simplicity and reasonable pricing.

The thesis has the following structure: Chapter 1 is an introduction to the topic with a description of the current trends and problems in the field of molecular diagnostics, the role of isothermal DNA amplification and biosensors in evolution of the field, the current state of the art and the objectives of this thesis. Chapters 2-5 report the work performed in order to achieve the specific objectives of this Doctoral thesis: the development of homogeneous multiplex isothermal system for detection of several pathogens in one pot, the use of solid-phase recombinase polymerase amplification using optical and electrochemical detection, surface chemistry enhancement, and the use of labelled dNTPs in the amplification process in order to simplify and shorten the time of the

assay. The conclusions and the future prospects are described at the end of the thesis.

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CHAPTER 1

Introduction

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INTRODUCTION

1.1. Molecular diagnostics

Molecular or nucleic acid-based diagnostic testing has grown significantly in recent years and has been largely influenced by different breakthroughs in basic understanding stemming from the knowledge gleaned from the Human Genome Project and post-HGP technologies including Next-Generation Sequencing. Molecular diagnostics itself combines laboratory medicine with the knowledge and technology of molecular genetics and has moved from complicated and manual to rapid and automated due to improvements in sample extraction, target amplification combined with sensitive and specific detection techniques.

1.1.1. DNA discovery and its influence on molecular diagnostics

DNA was first identified in the late 1860s by Swiss chemist Friedrich Miescher [1], who identified a "nuclein" as any group of phosphorus-containing proteins, that occur inside the nuclei of human white blood cells. The term "nuclein" was later changed to "nucleic acid" and to "deoxyribonucleic acid," or "DNA", due to the acidic nature endowed by the phosphate groups. Decades after Miescher's discovery, other scientists, like Phoebus Levene and Erwin Chargaff, carried out a series of research that revealed additional details regarding the DNA molecule, from primary chemical components to the ways in which they were linked [2]. These scientific discoveries provided by these pioneers provided the basis for Watson, Crick, Wilkin and Franklin's ground-breaking conclusion of 1953: that the DNA molecule exists in the form of

a three-dimensional double helix (*Figure 1.1*) [3], specifically elucidating that:

- DNA is a double-stranded helix, with the two strands connected by hydrogen bonds. A bases are always paired with T, and C are always paired with G.
- Most DNA double helices are right-handed. Only one type of DNA, called Z-DNA, is left-handed.
- The DNA double helix is anti-parallel, which means that the 5' end of one strand is paired with the 3' end of its complementary strand (and vice versa).
- The outer edges of the nitrogen-containing bases are exposed and available for potential hydrogen bonding [2].

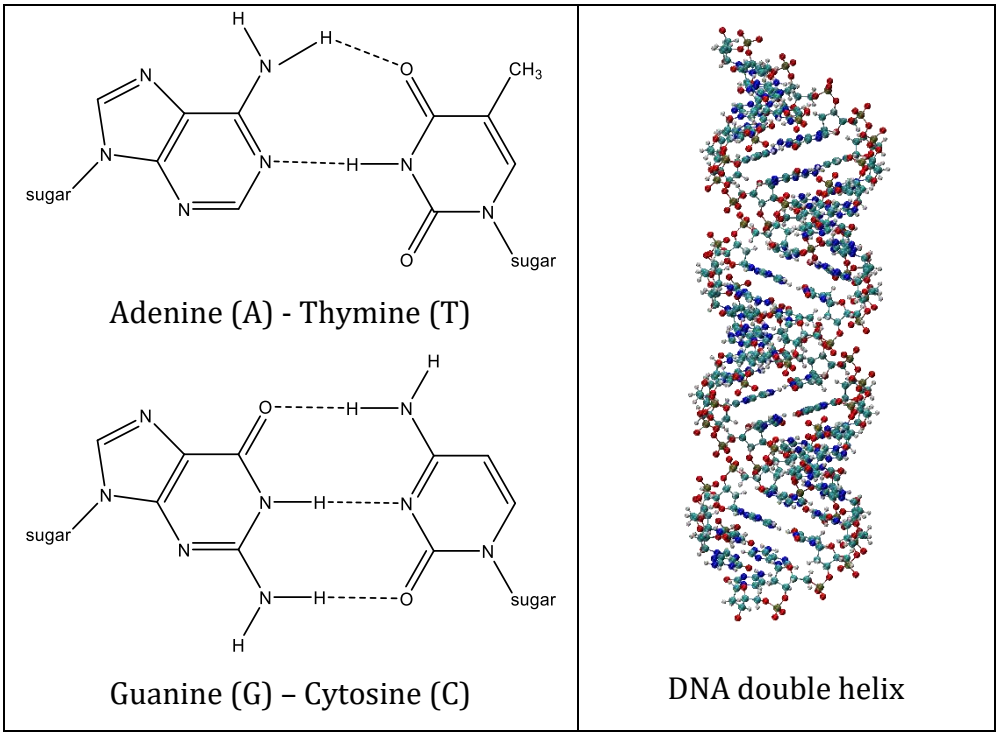


Figure 1.1 DNA structure

In 1977, two methods of DNA sequencing were reported, Sanger's chain-termination method and Maxam and Gilbert's chemical degradation method. In 1980, Sanger and Gilbert shared the Nobel Prize in Chemistry for the development of their respective sequencing methods[4,5]. The discovery of the polymerase chain reaction (PCR) by Mullis *et al.,* in 1986 [6] essentially revolutionised molecular diagnostics. PCR gave the ability to produce many copies of a target DNA, facilitating rapid analysis and direct detection of mutations. In 1990 the combination of these techniques resulted in the realisation of the human genome project (HGP), sequence the whole human genome in 15 years. On April 25th, 2003, 50 years to the day after the publication detailing the discovery of the structure of DNA, the decoding of the human genome was officially completed, resulting in the blueprint of the human body and the exact sequence of 3.2 billion bases. The total cost of the HGP was around $3 billion, or about $1 per base pair [7]. Since these crucial discoveries, progress in molecular diagnostics has proceeded at a startling speed and dynamic development in the field is still far from complete (*Table 1.1*).

Table 1.1 Molecular diagnostics milestones (modified from Patrinos et al., 2005 [8] and Demidov, 2003 [9])

Date	***Discovery***
1949	Characterisation of sickle cell anemia as a molecular disease
1953	Discovery of the DNA double helix
1957	Phosphonate synthesis of short oligodeoxynucleotides. Triple-stranded nucleic acid complexes are found
1958	Isolation of DNA polymerases
1960	First hybridisation techniques and inception of electrochemical DNA detection
1969	*In situ* hybridisation
1970	Discovery of restriction enzymes and reverse transcriptase
1975	Southern blotting

1977	DNA sequencing. First genome (bacteriophage φX174) is sequenced
1983	First synthesis of oligonucleotides
1984	Invention of PCR. Discovery of thermostable DNA polymerase
1985	Restriction fragment length polymorphism analysis
1986	Development of fluorescent in situ hybridisation (FISH)
1987/8	First triplex DNA probes
1988	Optimisation of PCR
1988/91	Origination of first DNA chip conceptions
1990s	Emergence of microelectromechanical systems and DNA nanotechnology
1992	Conception of real time PCR
1993	Discovery of structure-specific endonucleases for cleavage assays
1994	DNA topological labelling (padlock probes)
1995	First bacteria (Haemophilus influenzae) is sequenced
1996	First application of DNA microarrays
1998	Lab-on-a-chip (microfluidics) for DNA analysis
2000	Genomic sequence of fruit fly (Drosophila melanogaster)
2001	First draft versions of the human genome sequence
2001	Application of protein profiling in human diseases
2003	Official completion of the Human Genome Project

1.1.2. Molecular diagnostics and personalised medicine

Every day, millions of people with severe conditions are either not diagnosed or take medications that not only might not help them, but may indeed induce adverse side effects. Molecular diagnostics proposes the use of diagnostic testing to understand the molecular mechanisms of an individual patient's disease and will be essential in the delivery of safe and effective therapy for many diseases in the future [10,11]. The term "personalised medicine" was first coined in the 1990s, even though the concept is much older [12,13]. Molecular diagnostics in personalised medicine covers the following aspects [14]:

- Early detection and selection of safe and effective treatment via patient stratification
- Integration of molecular diagnostics with therapeutics
- Monitoring therapy as well as determining prognosis

The development of cost-effective and easy-to-use diagnostic tests is critical for early detection and monitoring of disease. The development process for a point-of-care (POC) diagnostic test from research to commercial implementation faces a wide range of problems, as detailed in Table 1.2.

Table 1.2 Diagnostics development: steps, barriers and solutions [15]

Diagnostics development	***Barriers***	***Solutions***
Discovery research **Proof-of-principle**	• Perceived lack of market • Poor understanding of the required test characteristics • Lack of funds for research and development • Lack of access to reagents and strains	• Market analysis • Defined product specifications • Strain/reagent/specimen bank
Laboratory evaluations of convenient samples	• Lack of access to clinical samples	• Specimen bank
Field trials in target populations	• Lack of access to trial sites	• Evaluation networks in disease-endemic countries
Product registration	• Lengthy regulation approval process	• Regulatory harmonisation
Usefulness/sustainability	• Lack of understanding of healthcare in developing countries	• Studies funded by the public sector to demonstrate feasibility, usefulness, sustainability and impact
Impact studies	• Lack of study sites • Lack of funds	
Patient access	• High cost of tests • Lack of policy for use	• Negotiated pricing • Inform policy makers of usefulness and health impact

Currently, personalised "patient oriented" medicine is garnering increasing interest. It allows tracking of a person's health at the most basic molecular level, detecting and measuring specific biomarkers that are related to specific diseases (*i.e.* genes, single nucleotide polymorphisms (SNP), messenger RNA (mRNA) or proteins they express). Either used individually, or in combination, information on disease predisposition and/or disease dissemination as well as prediction of response to specific therapies, can be obtained (*Figure 1.2*).

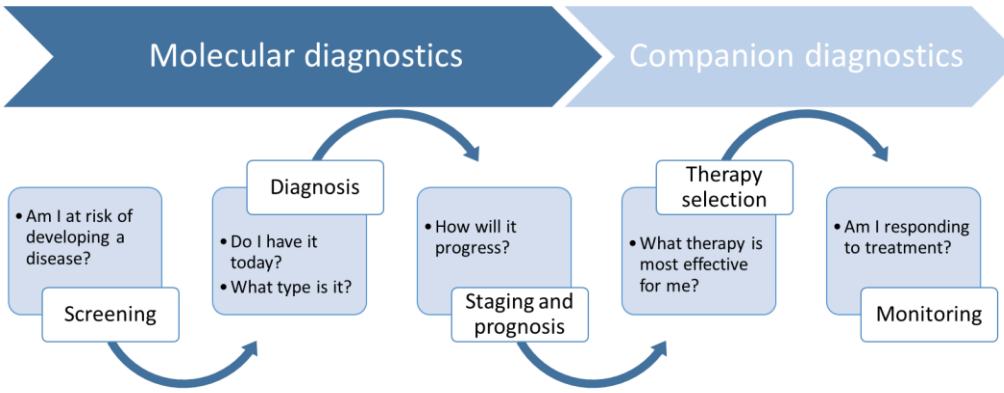


Figure 1.2 Tandem of personalised medicine and molecular diagnostics

1.2. Miniaturisation

In recent years, there has been an increased interest in the generation of POC molecular diagnostic devices. Miniaturisation of nucleic acid amplification methods offers several advantages such as the possibility to decrease required assay time, the use of lower sample volumes, reduction of instrumentation costs and the ability to perform complete analysis on a single device (*Figure 1.3*).

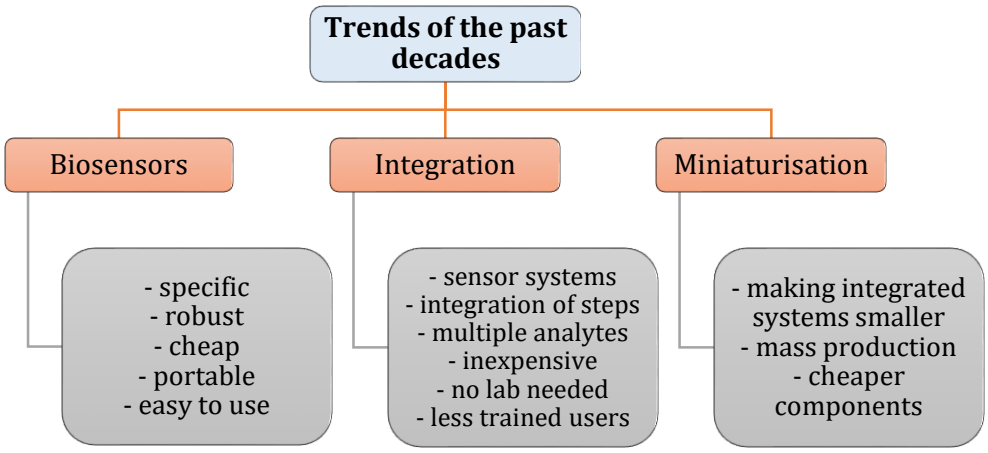


Figure 1.3 Trends in molecular diagnostics

1.2.1. Biosensors

The history of biosensors started with the development of enzyme electrodes by the L.L. Clark in 1962 [16]. Since then, multiple research groups from different fields have invested massive effort to create sophisticated, reliable and mature biosensing devices for applications in the fields of medicine, agriculture, biotechnology, as well as for military and bioterrorism applications (*Table 1.3*).

Table 1.3 Biosensor milestones (modified from Joshi, 2006 [17])

Date	***Discovery***
1916	First report on the immobilisation of proteins: absorption of invertase on activated charcoal
1956	Invention of the first oxygen electrode by Clark [18]
1962	First description of a biosensor: an amperometric enzyme electrode for glucose by Clark and Lyons [16]
1969	First potentiometric biosensor: urease immobilised on an ammonia electrode to detect urea
1970	Discovery of ion-sensitive field-effect transistor (ISFET) by Bergveld [19]
1975	Fibre-optic biosensor for carbon dioxide and oxygen detection by Lubbers and Opitz [20]
1975	First commercial biosensor for glucose detection by Yellow Springs Instruments

1975	First microbe-based immunosensor by Suzuki [21]
1976	First bedside artificial pancreas by Clemens [22]
1980	Fiber optic pH sensor for in vivo blood gases
1982	Fibre-optic biosensor for glucose detection by Schultz [23]
1983	Surface plasmon resonance (SPR) immunosensor by Liedberg [24]
1984	First mediated amperometric biosensor: ferrocene used with glucose oxidase for glucose detection [25]
1987	Launch of the blood glucose biosensor (MediSense)
1990	SPR-based biosensor by Pharmacia Biacore [26]
1992	Handheld blood biosensor by i-STAT [26]
1996	Launch of Glucocard
1996	Abbott acquires MediSense for $867 million
1998	Launch of LifeScan FastTake blood glucose biosensor
1998	Merger of Roche and Boehringer Mannheim to form Roche Diagnostics
2000	The now world-wide available painless blood glucose monitor FreeStyle was created
2001	LifeScan purchases Inverness Medical's glucose testing business for $1.3 billion

A biosensor, according to the International Union of Pure and Applied Chemistry (IUPAC), is "a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals" [27] (*Fig. 1.4*). In simpler terms, it is a device that utilises biological components to indicate the presence of a specific analyte. The indication may be qualitative, *e.g.* if the analyte is present or not; or quantitative, by measuring the quantity of the analyte [28].

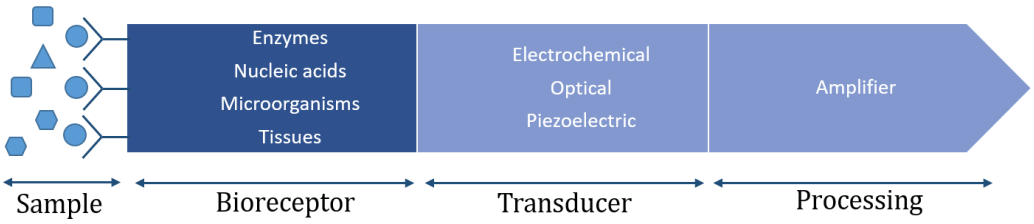


Figure 1.4 Schematic representation of a biosensor

A biosensor consists of two main parts: physical components (transducer and amplifier) and biological components (biological element). The classification of biosensors differs from one source to another but mainly they are classified according to the transduction methods they exploit, which can be categorised into five main classes: electrochemical, electrical, optical, gravimetric and thermal detection [29]. In this thesis we will mainly focus on electrochemical biosensors in combination with solid-phase Recombinase Polymerase Amplification. Optical biosensors are highly sensitive, rapid, reproducible, and simple-to-operate analytical tools [30,31]. Electrochemical ones have the added advantage of their relative simplicity. Inexpensive electrodes with simple electronics can be easily integrated to perform rapid measurements in miniaturised easy-to-use portable systems [32–34].

1.2.2. DNA amplification and detection techniques

In most cases, molecular diagnostics requires amplification of the target DNA because it is present in low concentrations. The Polymerase Chain Reaction [6] (PCR, Nobel prize in Chemistry, 1993) facilitated the rapid production of large amounts of DNA for subsequent analysis. A basic PCR set-up requires several components and reagents [35,36], including:

- DNA template to amplify
- DNA polymerase (*i.e.* heat-resistant *Taq* polymerase [37]) to polymerize new DNA strands
- Two DNA primers that are complementary to the ends of each of the sense and anti-sense strands of the DNA target, defining the sequence to be amplified

- Deoxynucleoside triphosphates (dNTPs) that are the building blocks for a new DNA strand synthesis
- Buffer solution for suitable chemical environment
- Bivalent cations (typically magnesium)
- Monovalent cations

The PCR reaction in generally carried out in a test tube or 96-well plate and placed in a thermal cycling machine that allows repeated cycles of DNA amplification to occur in three basic steps: denaturation, annealing and elongation[38]. Several methods have been developed for visualisation of the PCR products, including staining of the amplified DNA product with a chemical dye (*i.e.* ethidium bromide), which intercalates between the two strands of the duplex or labelling the PCR primers or nucleotides with fluorescent dyes (fluorophores) prior to PCR amplification. The most widely used method for analysing the PCR product is the use of agarose gel electrophoresis, based on the size-dependent separation of DNA products.

Quantitative real-time or qPCR provides additional information beyond just qualitative detection of DNA. It indicates how much of a specific DNA or gene is present in the sample. qPCR allows both detection and quantification of the PCR product in real-time, while it is being synthesized [39], either using fluorescent dyes that non-specifically intercalate with double-stranded DNA or sequence-specific DNA probes exploiting molecular beacon formats, the exonuclease digestion activity of Taq polymerase, or the distance-dependent transfer of energy between donor and acceptor dyes.

An alternative approach for the qualitative or quantitative detection of DNA takes advantage of detection via hybridisation. Since its

creation in 1971, enzyme-linked immunosorbent assay (ELISA) has been widely used in research and diagnostics as an optical detection system [40]. One of the possible ELISA approaches, commonly known as sandwich ELISA, simultaneously uses two antibodies or analyte-binding receptor proteins to capture the analyte of interest and to report target detection. Following this idea, Drolet *et al.* [41] developed a new detection method called enzyme-linked oligonucleotide assay (ELONA), in which the reporting antibody/protein of ELISA is substituted for a fluorescein-tagged nucleic acid specific for a particular target. The study showed that ELONA may be applied for quantification and its accuracy and specificity are comparable to the well-established ELISA. In ELONA approach, a probe is immobilised on the surface of a microtitre plate and the target DNA is detected in a sandwich assay, exploiting a secondary-labelled reporter DNA probe, for example via the use of a fluorescein-labelled oligonucleotide probe and an enzyme-labelled anti-fluorescein antibody, or directly HRP-labelled probe [42].

DNA biosensors (or genosensors) also exploit the mechanism of the preferential binding of complementary single stranded DNA (ssDNA) sequences to single stranded probes immobilised on a transducer (*Fig. 1.5*) [43,44].

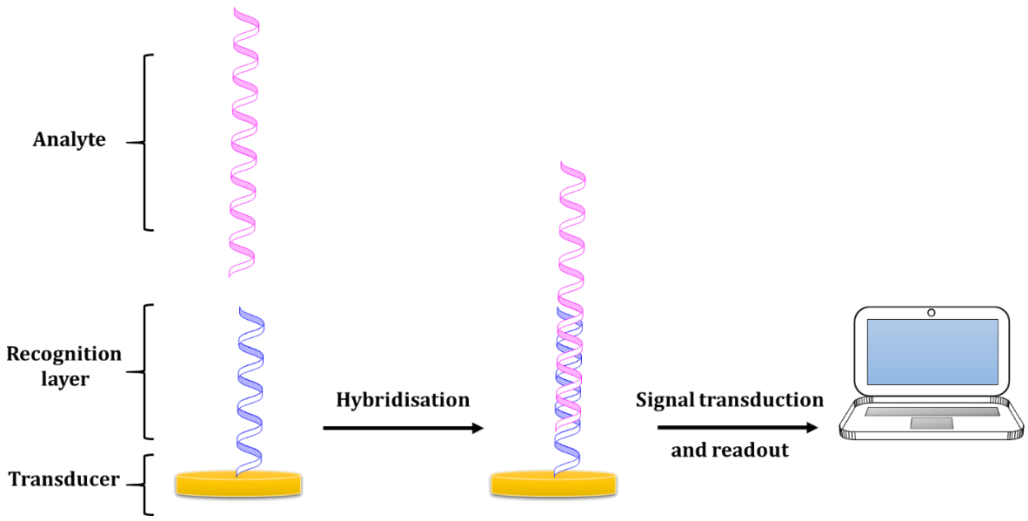


Figure 1.5 Schematic representation of a genosensor

A significant number of immobilisation strategies have been developed to tether the ssDNA probes to the surface of the transducer, ranging from simple physiosorption to direct and covalent immobilisation of biomolecules on surfaces using self-assembled monolayers (SAMs) or coupling to polymers. Each of these methods have their advantages and disadvantages. Physical adsorption of DNA on the surface is a widely used direct and simple immobilisation method that creates functional surfaces for bioanalytical applications. However, the mechanism for adsorption can suffer from limitations including random orientation and weak attachment of DNA to the surface. Weakly attached DNA probes can be easily removed by some buffers or detergents used in the assays. Problems of crowding effect and poor reproducibility as well as high background signals from non-specific interactions can result in false detection results. Covalent attachment by creating a SAM is a more robust method than physical adsorption. SAMs offer better stability and higher binding strength, provide uniform and high coverage structures, and reduce random orientations, all of which contribute to an improved

reproducibility and stability of the monolayers [45,46]. Due to these advantages, SAMs are often used for the development of biosensors with electrochemical, piezoelectric, or optical detection. Optical sensors often use glass or silica substrates and noble metals are mainly used for electrochemical sensors [47,48].

Silanisation of hydroxyl-terminated substrates (silica or glass) is an effective method for the immobilisation of biomolecules. It is a chemical modification of the substrate with self-organising silane monolayers. The general mechanism of silanisation consists of three main steps [49]. In a first step, the silanes form silanetriols by hydrolysis in the presence of water, on the surface or in the solvent. These silanetriols attach themselves by physiosorption via hydrogen bonds onto the substrate surface. Subsequently, the silanol groups react with the free hydroxyl groups on the surface according to a S_N2 reaction mechanism (*Figure 1.6*).

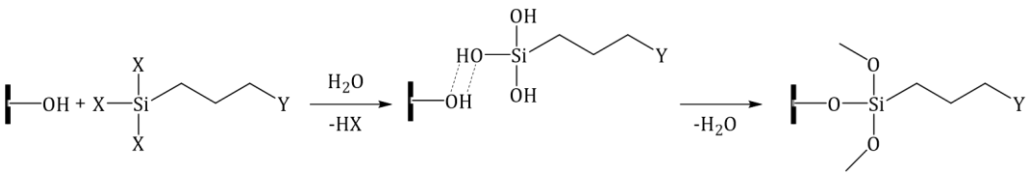


Figure 1.6 Depictions of silanisation mechanism

A DNA immobilisation strategy for electrochemical biosensors exploits a thiol-metal interactions for covalent binding of biomolecules on gold surfaces. The thiol, disulphide and sulphide groups have a strong affinity towards the noble metal surfaces allowing the formation of covalent bonds between the sulphur and gold atoms (*Figure 1.7*).

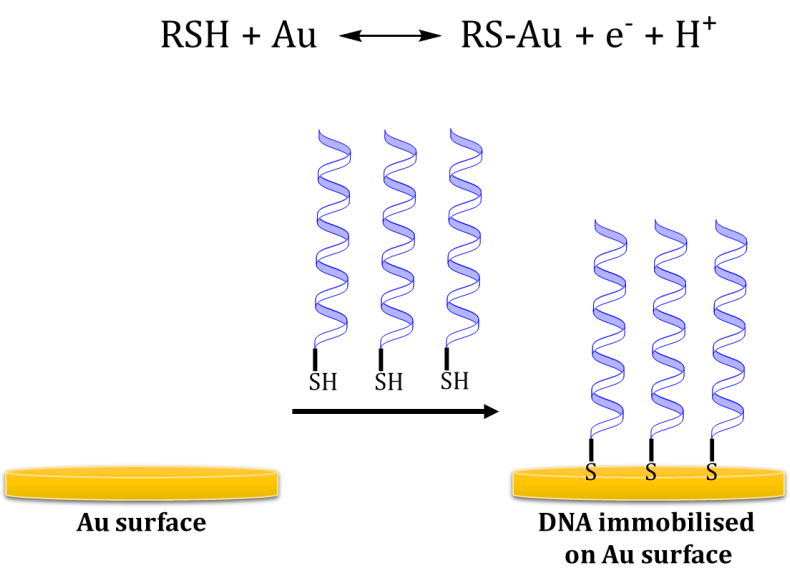


Figure 1.7 DNA immobilisation on Au (Gold) surface

In this work, we use mixed SAMs of thiolated DNA probes and short chain alkanethiols. This approach, first reported by Herne and Tarlov in 1997 [50], takes advantage of a two-step method:

1) exposure of gold electrode by a µM solution of thiolated nucleic acid probe;
2) exposure to a mM solution of a short-chain alkanethiol, also called "backfiller"

This system provides good packing density, the "backfiller" can prevent a possible non-specific adsorption and improving probe orientation.

However, simultaneous co-immobilisation of thiol-modified DNA with a thiolated spacer molecule to form a mixed monolayer is also possible and often results in more-reproducible surfaces. In this approach, the probe surface density is controlled by optimisation of the

molar ratio between the thiolated probe and the alkanethiol co-immobiliser in the deposition solution [51].

1.2.3. Label and label-free detection methods

A good detection method generally should provide a high signal-to-noise ratio and good reproducibility. Furthermore, the detection methods should have low instrumentation costs, be rapid and robust, while producing reliable and accurate qualitative or quantitative results. Currently, the majority of developed DNA detection methods require the application of a label to detect the target, but there are an increasing number of label-free strategies for both optical and electrochemical genosensors. Even though techniques using labels e.g. enzyme are highly sensitive, the requirement for the addition of substrate incurs additional assays steps and inherent cost [52].

Several strategies of labelled detection are highly exploited in DNA detection [53]:

- *electroactive labels* are electron mediators that enable the reversible exchange of electrons with the electrode's surface (*e.g.* [Os(5,6-dimethyl-1,10-phenanthroline)2Cl]$^{2+}$ [54], Co(phen)$_3^{3+}$ [55], Co(bpy)$_3^{3+}$, Fe(CN)$_6^{3-/4-}$ [56], methylene blue [57]). The mechanism of these labels is based on selective interaction of these substances with the double stranded DNA (dsDNA).This method is simple and does not require chemical modification of the DNA target
- *redox-active labels* are DNA-intercalating and groove-binding compounds offering much higher affinity for the resulting hybridised molecule compared to the single stranded probe

(*e.g.* fluorescent labelled probes [58], ethidium bromide [59], Hoechst 33258 [60], gold nanoparticles with doxorubicin [61], anthraquinone [62])

- *enzyme labels* may be linked to the target through biotin–avidin interaction or to a reporter oligonucleotide. They provide signal amplification and consequently a higher sensitivity, but require addition of substrates (*e.g.* horse-radish peroxidase (HRP) [63], alkaline phosphatase (AP) and glucose oxidase (GOD) [64]), and a preferred and simpler methodology would either be label-free or reagentless

The first developed label-free method aimed to detect changes in the physical properties of the recognition layer [65], including measurement of the alleged natural electroactivity of the nucleotides present in DNA [66]. In the early 1960s, Palecek and his group discovered the electroactivity of the nucleotide bases. Guanine has been shown to be the most easily oxidised one and has been used for indicator-free hybridisation detection [67]. A signal reduction in guanine oxidation was used as an indicator of target hybridisation, but this approach is not suitable for containing guanine targets. To overcome this limitation, Wang *et al.* substituted guanine present in the sequence of the capturing probe with inosine bases that can form hydrogen bonds with cytosine and DNA hybridisation was detected via oxidation of the guanine bases in the target [68].

Electrochemical impedance spectroscopy (EIS) is considered as a powerful tool, for the label-free detection of DNA hybridisation. These sensors are based on the modulation of the blocking ability of an electrode modified with a probe by the target DNA. The probe is

immobilised via a self-assembled monolayer, a conducting polymer film or a layer of nanostructures for selective hybridisation with target DNA. Faradaic EIS is normally performed in the presence of a redox active indicator ($[Fe(CN)6]^{3-/4-}$). The rate of charge transfer is changed due to the presence and quantity of selectively hybridised target [69,70].

Alternatively, transduction of the surface hybridisation event can be optically measured via plasmon resonance methods in multiple formats (*e.g.* surface-enhanced Raman scattering [71], Mach-Zehnder [72] and resonant interferometers [73,74]).

One of the possible reagentless detection methods uses redox modified dNTPs that are incorporated during the elongation process as building blocks of DNA. DNA containing functionalised nucleobases have immense potential for applications in chemical biology, nanotechnology, material science, as well as bioanalysis. Since the 1990s, there have been multiple reports on the synthesis of labelled dNTPs including amino and thiol [75], imidazoyl moieties [76] and they serve for the preparation of functionalised DNA. More recently, other redox-labelled dNTPs have been reported including amino- and nitrobenzene [77], ferrocene [78], Ru- or Os(bpy) complexes [79], alkylsulfanylphenyl [80], anthraquinone [81], benzofurazane [82], azidophenyl [83] and polyoxometalates [84] (*Fig.1.8*).

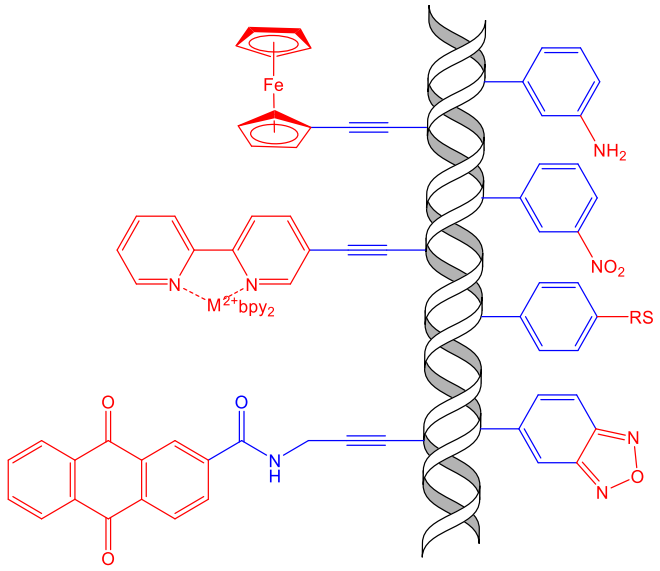


Figure 1.8 Examples of dNTPs modified with redox molecules

Modified dNTPs can be prepared using two approaches. In the classical approach, functionalised dNTPs are obtained by cross-coupling of a halogenated 2'-deoxynucleoside followed by triphosphorylation of the resulting modified nucleoside to obtain modified dNTPs. The second and more straightforward approach is a direct aqueous cross-coupling reaction of a halogenated dNTP. In 1967, Yoshikawa *et al.* [85] described the first method for the synthesis of dNTPs. Briefly, the procedure involves selective 5'-mono-phosphorylation of an unprotected nucleoside with phosphorous oxychloride yielding a 5'-phosphorodichloridate intermediate that is further treated *in situ* with tributylammonium pyrophosphate to yield a cyclic triphosphate, which is subsequently hydrolysed to the desired dNTP. Ludwig and Eckstein [86] reported 5'-O-triphosphorylation of 2'-deoxynucleosides using salicyl chlorophosphite in the first step. The cyclic intermediate is further oxidised by iodine and

hydrolysed to the desired dNTP. This approach has higher specificity compared to the Yoshikawa method.

The second approach is the direct synthesis of dNTPs by aqueous cross-coupling reactions. Cross-coupling reactions are in general very tolerant to most of reactive functional groups. This methodology appeared due to the development of water soluble phosphine ligands [87]. Several coupling methods exist: Sonogashira coupling [88], Suzuki–Miyaura coupling [89] and Heck reaction [90].

Modified dNTPs are utilised in the enzymatic synthesis of modified DNA. Generally, in order to preserve the Watson–Crick pairing and minor-groove interactions, the modifications must be attached to positions which point out to the major groove [91]. This condition is essential for efficient and specific incorporation by DNA polymerases. In pyrimidines, for example, the best modification site is linked at position 5. Other position modifications (position 8) show to be a poor substrate for DNA polymerase and reverse transcriptase [92].

There are several methods for polymerase synthesis of DNA. One of the most basic ways to produce short dsDNA modified in one strand is primer extension (PEX). This is an isothermal reaction suitable for particular types of DNA polymerases such as the Klenow fragment of *Escherichia coli*. PCR is used for the preparation of longer DNA with a higher level of density of modifications in both strands. The temperatures and times of individual steps depends on the sequence, length, and sequence of primers, the DNA polymerase used, and the types of modifications of dNTPs, so every modification requires an individual optimisation of conditions. Since the PCR also requires the polymerase to be able to read through modified template, the PCR reaction often fails for

dNTPs modified by bulkier groups. Many modified dNTPs might be enzymatically incorporated by several various DNA polymerases (Taq, Sequenase and *Bst*). However, the KOD XL proved to be the most robust for the enzymatic synthesis of modified DNA and became the polymerase of the first choice.Various modified dNTPs found several applications in modern chemical biology, each of them offering their own advantages and limitations. For example, ferrocenes are prone to oxidation by air, the dNTPs bearing bulky inorganic [Os(bpy)3] are poor substrates for DNA polymerases, and the redox potential of Os^{2+} is close to that of 7-deazaguanine base [81,93]. Nevertheless, the choice of redox labels is still wide enough, so the proper label can be selected for each application and system.

1.2.4. Characteristics of the ideal diagnostic test — ASSURED

The ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable to end-users) criteria, outlined by the World Health Organisation (WHO) for developing countries, provide a good framework for evaluating POC devices for resource-limited settings. Tools that satisfy the ASSURED criteria primarily aim to provide same-day diagnosis and facilitate immediate decision-making [94].

Most of the currently developed POC tests use immunochromatographic strips to detect antigens or antibodies in a dipstick or lateral-flow format. Some tests, such as the direct agglutination test (DAT) are based on the visualisation of antigen–antibody lattice formation, either by coating red blood cells or latex particles with the antigen or antibody, or by entrapping charcoal or dye

particles in the reaction to allow a positive result to be visualised [95,96]. These tests are inexpensive, reproducible, and simple to perform, produce rapid visual readouts and generally require no equipment. They are useful in resource-limited countries because they do not require electricity for equipment or refrigerators (for the storage of reagents). However, most of POC tests developed still fail to meet the ASSURED needs of disease-endemic areas due to one or more parameters. A simple example of the requirements for an HIV ASSURED test established by the World Health Organisation (WHO) in 2012 is detailed in *Table 1.4* [94].

Table 1.4 ASSURED characteristics (WHO) and examples of target specifications for the evaluation of point-of-care devices

Characteristic	***Target Specification***
Affordable	Less than $500 per instrument, less than $10 per test
Sensitivity, Specificity	Lower limit of detection: 500 HIV RNA copies per ml of blood
User-friendly	1-2 days training, easy to use
Rapid and robust	< 30 minutes for diagnosis; Shelf-life > 1 year at room temperature
Equipment-free	Compact, battery-powered, on-site data analysis, easy disposal, easy sample handling, no cold chai
Deliverable	Portable, hand-held

1.3. Isothermal amplification techniques

Whilst PCR has revolutionised molecular diagnostics, facilitating the rapid amplification of DNA, producing billions of copies of a specific DNA fragment or gene, that can be either be quantitatively detected in real time, or subsequently analysed by any of a battery of techniques including detection via hybridisation and gel visualisation, there are several limitations of PCR for implementation in a POC device [37,97], including the need for thermal cycling, risk of contamination, the

relatively high cost of equipment and inherent requirement for a power source / electricity and a need for trained personnel.

To address the true implementation of nucleic acid amplification tests (NAAT) at the point-of-care/need, isothermal amplification techniques have been developed, such as loop mediated isothermal amplification (LAMP), nucleic acid sequence based amplification (NASBA), helicase-dependent amplification (HDA), strand displacement amplification (SDA), rolling circle amplification (RCA) and recombinase polymerase amplification (RPA), thus avoiding the need for thermal cycling apparatus [98,99].

1.3.1. Loop-mediated isothermal amplification (LAMP)

LAMP is a specific, relatively simple, rapid and cost-effective isothermal nucleic acid amplification method first described by Notomi *et al.* [100] in 2000. LAMP takes advantage of strand-displacing *Bacillus stearothermophylus* (Bst) DNA polymerase and 4-6 specifically designed primers (2 of which are 'fold back' primers for stem-loop formation) that recognise 6 distinct sequences on the target DNA [101]. The target amplicon is generated in a one-step amplification reaction using isothermal conditions (60-65 °C) in a reasonably short time (45-60 min). Two additional performance enhancement strategies have been reported to increase the speed and analytical sensitivity of LAMP by using additional set of loop primers and/or an initial melting step (95 °C) [102,103]. Even though LAMP requires a careful design of multiple complex primers, it is still one of the most widely used and studied isothermal amplification techniques due to its high-level of specificity, sensitivity (six DNA copies) and tolerance to various inhibitors present in clinical samples [100,102].

1.3.2. Nucleic acid sequence-based amplification (NASBA)

Nucleic acid sequence-based amplification**,** NASBA, and the similar methods, transcription mediated amplification (TMA) [104] and self-sustained sequence replication (3SR) [105] are alternative isothermal transcription-based amplification systems. NASBA is specifically designed for the detection of RNA targets but in some cases can be applied to DNA targets. Amplification occurs at a constant temperature of 41 °C with an initial strand separation step of 95 °C in the case of dsDNA and 65 °C in the case of RNA amplification[106]. The system uses three enzymes (avian myeloblastosis virus reverse transcriptase, RNase H and T7 DNA dependent RNA polymerase) producing single-stranded RNA as the main amplification product [107]. The assay time varies from 90 min to 3 hours. Transcription-mediated amplification (TMA) differs very slightly from NASBA and 3SR: instead of using three separate enzymes, the role of RNase H is carried out by the reverse transcriptase, so that only two enzymes are involved [108–110].

1.3.3. Helicase-dependent amplification (HDA)

Helicase-dependent amplification, HDA, reported for the first time in 2004 [111], uses a simple reaction scheme for isothermal amplification that more closely resembles *in vivo* DNA replication. This process exploits the unwinding activity of a DNA helicase in the presence of adenosine triphosphate(ATP) [112]. The enzyme helicase separates the two strands and the displaced DNA strands are prevented from reforming the helix by single-stranded DNA binding proteins. A set of forward and reverse primers binds to the unwound DNA, followed by polymerase mediated

elongation, and the process is repeated cyclically. Exponential amplification can typically be achieved in 60–120 min at a single constant temperature of 60-65 °C, with no requirement for an initial DNA melting step.

1.3.4. Strand Displacement Amplification (SDA)

Strand Displacement Amplification**,** SDA, first described in 1992, [113] relies on a strand-displacing DNA polymerase (Bst DNA polymerase, Large Fragment or Klenow Fragment (3'→5' exo-), to initiate replication. SDA uses four different primers, one of which contains a restriction site (a recognition sequence for HincII exonuclease) and anneals to the DNA template, followed by Bst DNA polymerase-mediated elongation. Each SDA cycle consists of primer binding to a displaced target fragment, extension of the primer/target complex by the strand-displacing DNA polymerase, nicking of the resultant hemiphosphothioate HincII site, dissociation of HincII from the nicked site and extension of the nick and displacement of the downstream strand by DNA polymerase [114]. This method can be performed at a various temperatures (37 °C to 70 °C). In one single reaction, 10^9 copies of target DNA can be produced in less than an hour.

1.3.5. Rolling circle amplification (RCA)

Rolling circle amplification, RCA,[115] is an isothermal nucleic acid amplification method which utilises the strand displacement activity of the Phi29 bacteriophage DNA polymerase [116] acting on circular DNA targets. The basic RCA reaction (linear or single primer RCA), is initiated by a primer annealing to a circular ssDNA, following an initial melting

step. The Phi29 DNA polymerase then elongates a new strand of the circular template eventually completing a loop and reaching the point of initiation. Strand displacement activity allows the newly forming strand to continuously displace the previously generated strand as polymerisation advances. The isothermal system operates at a constant temperature (30–60 °C) with the process being completed in 30–90 min [117] and can start with as low as 10 copies of DNA [118].

1.3.6. Recombinase polymerase amplification (RPA)

Of all the isothermal techniques developed to date, recombinase polymerase amplification, RPA,[119] deserves special attention. It operates at a stable isothermal temperature (37–42 °C), without a need for an initial DNA melting step, and amplification can be achieved in just 5-10 min. This technique is the most appropriate for incorporation in an ASSURED diagnostic device (*Table 1.5*) [98,120–123].

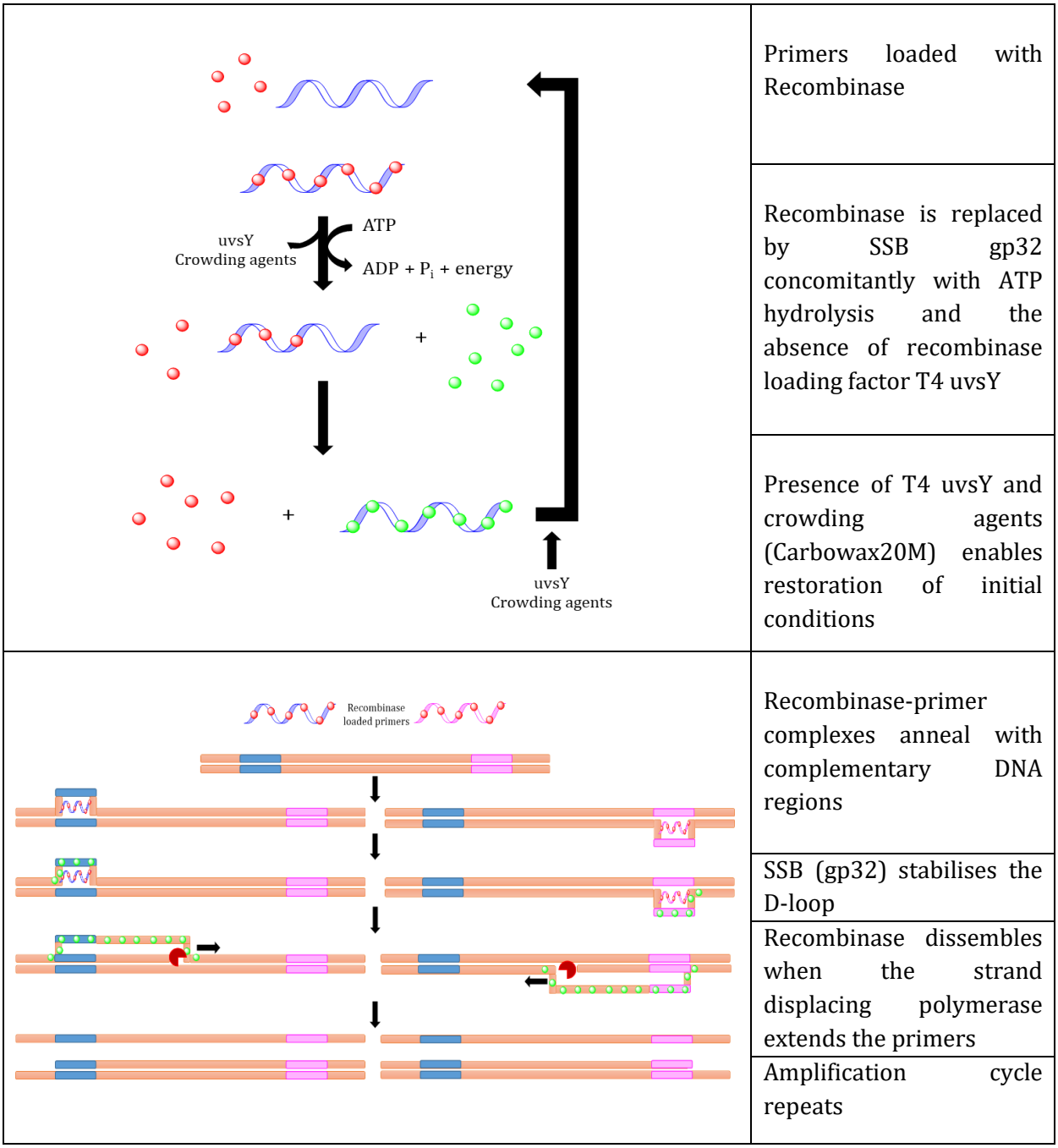
The mechanism of RPA involves the use of recombinase proteins that are present in living cells and are essential for the repair and maintenance of DNA [124–127]. The process is initiated when T4 uvsX (Recombinase) and T4 uvsY (loading factor) form a nucleoprotein filament with primers and probes. This complex scans the dsDNA target for homologous sequences. When the homologous sequence is found, the recombinase proteins facilitate a strand-invasion process and the formation of a D-loop. To prevent the closure and re-hybridisation of the initial dsDNA template, the displaced strand is stabilised by the single strand binding proteins (SSB) gp32. Finally, the recombinase dissembles when the strand displacing *Bsu* polymerase extends the primers. Following the first round of amplification, 2 duplexes are generated and

consequently used for another round of RPA and exponential amplification is reached by repeating the RPA cycle, which is described as self-perpetuating until exhaustion of the phosphocreatine pool [128] (*Figure 1.9*).

Table 1.5 Comparison of currently available isothermal amplification techniques

Method	*Preferred template*	*Amplicon type*	*Performance (upper)*	*Analytical sensitivity*	*Reaction temperature (initial temp) (°C)*	*Required enzymes*	*Primers required/ Primer design*	*Interventions (temperature /reagent additions)*	*Multiplexa-bility*	*Detection*
LAMP	ssDNA	Concaten-ated DNA	10^9-fold in 60 min	~5 copies	60–65(95)	1: DNA polymerase	4–6/ complex	2	Yes	Gel electrophoresis, turbidity, intercalating DNA dye, fluorescence probe, real time
NASBA	RNA	RNA	Fluorescent detection within 60 min	1 copy	41 (65/95)	2 or 3: reverse transcriptase and RNA polymerase (RNase H)	2/ simple	2	Yes	Gel electrophoresis, ELISA, fluorescence, real time, electrochemilu-minescent
HDA	dsDNA	dsDNA	10^{10}-fold amplification in ~100 min	1 copy	64	2: DNA polymerase and helicase	2/ simple	1	2 plex plus Internal control	Intercalating DNA dye, fluorescence probe, lateral flow strip

SDA	ssDNA	dsDNA	10^7-fold in 2 h	10 copies	37 (95)	2: DNA polymerase and NEase	2 DNA/RNA chimeric primers + 2 bumper primers/ complex	2	No	Gel electrophoresis, fluorescence, real time
RCA	Circular ssDNA	Concate-nated ssDNA	10^9– fold in 90 min	10 copies	30–65(95)	2: DNA polymerase and NEase	1/ complex	2	No	Gel electrophoresis, fluorescence, real time
RPA	dsDNA	DNA	Detection in 20 min	1 copy	37–42	2: DNA polymerase and recombinase	2/ simple	1	Yes	Gel electrophoresis. proprietary fluorescence probe, colorimetry, DVD platform, SERS, lateral flow



Primers loaded with Recombinase

Recombinase is replaced by SSB gp32 concomitantly with ATP hydrolysis and the absence of recombinase loading factor T4 uvsY

Presence of T4 uvsY and crowding agents (Carbowax20M) enables restoration of initial conditions

Recombinase-primer complexes anneal with complementary DNA regions

SSB (gp32) stabilises the D-loop

Recombinase dissembles when the strand displacing polymerase extends the primers

Amplification cycle repeats

Figure 1.9 Schematic outline of RPA process

Since RPA was developed by O. Piepenburg *et al.,* in 2006 [119], it has garnered immense popularity and interest in the scientific world, resulting in a significant and ever-increasing amount of publications in the field of molecular diagnostics. By August 2017, over 170 peer-reviewed reports detailing diverse aspects and different applications of the method have been published (*Figure 1.10*).

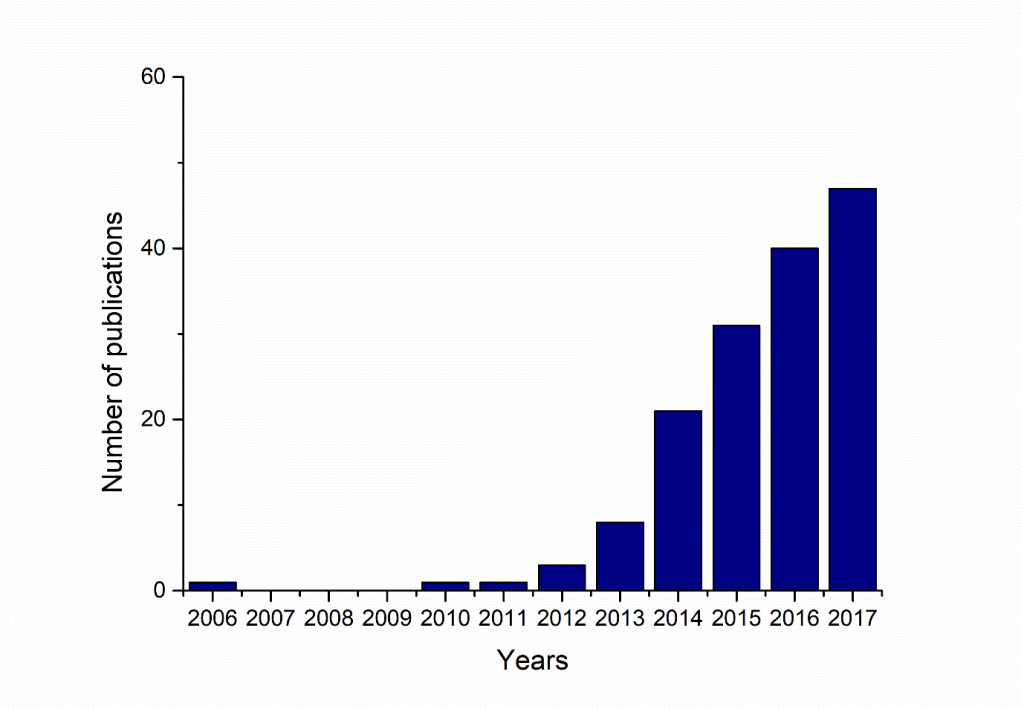


Figure 1.10 Peer-reviewed publications for Recombinase Polymerase Amplification, 2006-2017

RPA has been used for the detection of a wide number of targets, including bacteria [129–134], parasites [135,136], fungi [137], cancer cells [138–142],viruses [130,143–145] and GMOs [146–151], and can be used to effectively amplify both DNA and RNA templates. The limit of detection and amplification time varies, but may be dependent on the sequences of the target, the primers, the amplicon size and the type of sample used. Both,

end point and real time detection has been reported. The major detection methods reported are lateral flow assays [119,152–159] and real-time fluorescence detection systems [129,132,160–168]. Lateral flow assays offer a cost-effective solution with simple visual detection system where a qualitative end-point assay is sufficient. On the other hand, fluorescence detection systems can provide real-time monitoring where quantitative measurement is required but it needs specialised read-out instrumentation.

Other end point detection systems reported include agarose gel detection [169–174], colorimetric assays [149,175–177], ring resonators [178,179], solid-phase DVD platforms [148,180,181], electrochemical methods [177,182–186] and surface enhanced Raman spectroscopy (SERS) detection [139,141,187]. A full review of the published assays on RPA is displayed in Annex 1

1.4. Multiplexing RPA

Although RPA singleplex assays (*i.e.* assays to measure a single analyte) are robust and represent the majority of RPA research reported to date, it is also of great interest to simultaneously amplify and detect more than one target. To date, several RPA multiplex assays have been reported for the detection of several targets using various detection systems. Both, DNA and RNA targets are successfully used in these developed assays.

In comparison to other isothermal techniques, RPA is one of the simplest due to its simplicity in primer and quantity of primers. In comparison, LAMP requires a set of 4-6 primers [188] that can both enhance selectivity and promote cross reactivity. In addition to the primer design, RPA uses dsDNA as an initial template that avoids the need of pre-melting

or ssDNA generation. Constant reaction temperature (37 – 42 °C) is a further advantage of RPA because in resource-limited environment, RPA can be done using the temperature of body to initiate the reaction.

Piepenburg *et al.* [119] were the first ones who applied RPA for multiplexed detection of three variants of the staphylococcal cassette chromosome mec (SCCmec) elements in MRSA. The multiplex mixture contained 4 primers, 3 probes and an internal control in a single reaction. The assay was able to detect as low as 10 genomic copies of the MRSA in 30 min using real time and lateral flow detection systems. Subsequently, more real time fluorescent detection assays were developed for detection of cancer genes [189], bacteria [162,190,191], and viruses [192,193] as well as the use of portable platforms, including lateral flow [146,194], digital versatile discs [147,195] and even Smartphones [181]. In 2017, Song *et al.* reported a combination of RPA and LAMP called RAMP, for the detection of ≥16 targets in only 40 minutes of reaction. A home-made microfluidic chip was used as a prototype POC device for multiplex detection of DNA and RNA targets [196]. However, not all of the reported systems are truly multiplex, and some of the developed methods are in reality parallelised singe reaction assays [195] (*Table 1.6)*.

Table 1.6 Examples of multiplexing assays using RPA

Target	Template	Biological sample	Performance (time and LOD)	Amplification T (°C)	Detection method	Multiplexing	Ref.
Botrytis cinerea, Pseudomonas syringae, Fusarium oxysporum	DNA	Infected leaves	1.9 fmol in 20 min	37	SERS	Yes	[197]
Campylobacter coli and *Campylobacter jejuni*	DNA	Eggs, chicken meat, chicken broth	1-103 CFU/ml in 20 min	45	Real time fluorescence	Yes	[198]
Cancer fusion genes	RNA	Urine	1000 copies in 15 min	37	Real time fluorescence	Yes	[189]
Ebola virus	RNA	Clinical samples	10^7 copies/μL in 20 min	40	Real time fluorescence Paper microfluidics device	Yes	[192]
ESKAPE Bacterial Pathogens	DNA	Cell culture	10 copies in 30 min	37	Fluorescence Microfluidic cartridge- based B-chip	Yes (parallelised)	[199]
Genetically modified organisms (GMOs)	DNA	Food samples	7 μg g-1 in 40 min	37	Digital versatile discs (DVD)	Yes (parallelised)	[147]
Genotyping	DNA	Buccal smear samples	103 pg in 40 min	37	Colorimetry	Yes (parallelised)	[200]
Genuity Roundup Ready 2 Yield (RR2Y) material in soybean (Glycine max)	DNA	Seed Extracts	10 copies in 15-20 min	39	Lateral flow assay	Yes	[146]

Giardia, Cryptosporidium, and *Entamoeba*	DNA	Spiked Stool	368 – 425 copies in 35 min	37	Lateral flow assay	Yes	194
Group B *Streptococci*	DNA	Vaginal, anal samples	20 copies in 30 min	39	Real time fluorescence	Yes	190
Hepatitis B, C HIV	Viral DNA/RNA	Whole blood	1000 viral genetic copies per millilitre in 10-30 min	37	Quantum dot barcode Smartphone optical device	Yes	193
Human adenovirus 41, Phi X 174, *Enterococcus faecalis*	DNA	Plasmid standards	35 GU/μL, 1 GU/μL, 5 × 103 GU/μL in 40 min	39	Chemiluminescence Flow-based chemiluminescence microarray	Yes	201
Klebsiella pneumoniae carbapenemase New Delhi metallo-β-lactamase	DNA	Cell culture	10 copies in 25 min	39	Real time fluorescence Digital Microfluidic Device	Yes	191
Leishmania infantum	DNA	Dog blood	0.8 parasites per mL of blood in 10 min	37	Chronoamperometry	Yes	183
Methicillin resistant *Staphyloccus aureus* (MRSA)	DNA	Genomic DNA	10 copies in 30 min	37	Agarose gel Lateral flow	Yes	119
miRNA	RNA	Urine	40 copies/pg in 15 min	37	Real time fluorescence	Yes	202
MRSA	DNA	Nasal and groin swabs	N/A in 20 min	39	Real time fluorescence	Yes	162
MRSA	DNA	N/A	<10 copies in <20 min	37	Real time fluorescence Foil-based centrifugal microfluidic cartridge	Yes (parallelised)	203

Neisseria gonorrhoeae, Salmonella enterica, MRSA	DNA	Genomic DNA	10-100 copies in 40 min	38	Fluorescence In-house produced biochips	Yes	204
Peanut, GMO, *Salmonella* spp., *Campylobacter* spp.	DNA	Reference strains, certified reference materials	48.7-900 ng in 45 min	37	Digital versatile discs (DVD)	Yes (parallelised)	195
Prostate cancer cells	RNA	Urine	100 copies in 15 min	41	SERS	Yes	139
Prostate cancer cells	RNA	Cell culture, urine and tumor samples	100 copies in 20 min	41	SERS	Yes	205
Pseudomonas syringae, Fusarium oxysporum, Botrytis cinerea, Fusarium oxysporum cubense, Cucumber mosaic virus, Bovine Herpesvirus 1, *Escherichia coli*, HIV, *Plasmodium falciparum*, *Mycobacteria Tuberculosis*	DNA/RNA	Stems, leaves, cells, blood cultures, water	N/A in 15 min	37	Agarose gel Naked eye	Yes	169

Schistosoma mansoni, HIV-1 clade B, S. hematobium, *Plasmodium falciparum*, *Schistosoma japonicum*, *Brugia malayi*, *Strongyloides stercoralis,* drug-resistant *Salmonella*, Zika virus (ZIKV)-America strain (mex 2–81, Mexico), ZIKV-Africa strain (MR 766, Uganda), human papilloma virus (HPV)-58, HPV-52, HPV-35, HPV-45, HPV-18, and HPV-16	DNA/RNA	Mice serum, spiked human serum and whole blood, spiked urine samples	0.5 fg in 20 min RPA + 15-20 min LAMP	37 (RPA) 60-65 ºC(LAMP)	Colorimetric or fluorescent dye and Smartphone	Yes	196
Salmonella	DNA	Food and clinical samples	6-30 CFU/ml in 40 min	37	Digital versatile discs (DVD)	Yes (parallelised)	180
Salmonella enterica, *Escherichia coli O157:H7*, *Vibrio parahaemolyticus*	DNA	Milk samples	4 cells in 30 min	39	Real time fluorescence	Yes (parallelised)	206

1.5. Model targets

In the work reported herein, we developed assays for the rapid detection of biological agents that could contribute to both personalised medicine and environmental protection; as well as for the detection of toxic microalgae that is an important for seawater and food safety.

1.5.1. Bioterrorism: biological agents

Biological weapons may include any microorganism (such as bacteria, viruses, or fungi) or toxin (poisonous molecules produced by microorganisms) that can be used with the intent to kill or incapacitate humans, animals or plants as an act of war. Throughout history, acts of bioterrorism ranging from a simple hoax to the actual use of these biological weapons have resulted in thousands of deaths *(Table 1.7)*. Today, the use of biological weapons is prohibited under customary international humanitarian law [207], as well as a variety of international treaties, most importantly *The United Nations Convention on the prohibition of the development, production, and stockpiling of bacteriological and toxin weapons and their destruction* [208], and the use of biological agents in armed conflict is a war crime [209].

Table 1.7 Examples of biological agent use during the past 2000 years (modified from Riedel, 2004) [210]

Time	***Event***
600 BC	Solon uses the purgative herb hellebore during the siege of Krissa
1155	Emperor Barbarossa poisons water wells with human bodies in Tortona, Italy
1346	Tartar forces catapult bodies of plague victims over the city walls of Caffa. Crimean Peninsula (now Feodosia, Ukraine)
1495	Spanish mix wine with blood of leprosy patients to sell to their French foes in Naples, Italy
1675	German and French forces agree to not use “poisones bullets”

1710	Russian troops catapult human bodies of plague victims into Swedish cities
1763	British distribute blankets from smallpox patients to Native Americans
1797	Napoleon floods the plains around Mantua, Italy, to enhance the spread of malaria
1863	Confederates sell clothing from yellow fever and smallpox patients to Union troops during the US Civil War
World War I	German and French agents use glanders and anthrax
World War II	Japan uses plague, anthrax, and other diseases; several other counties experiment with and develop biological weapons programs
1951	Countries started programs to study how to protect their troops from biological weapons
1961	Biological weapon production ceases in U.S.
1972	Signing of the Biological Weapons Convention, which states that no country can stockpile biological weapons for military purposes. Russia and Iraq have violated this agreement.
1978	One of the first incidences of bio-terrorism. Georgi Markov was stabbed and infected with ricin
1979	A biological weapons plant mistakenly released anthrax spores. The anthrax killed an unknown amount of people. A laboratory confirmed that there were four different strains of the anthrax spore
1981-1993	The apartheid regime in South Africa runs Project Coast, which designs and uses a variety of chemical and biological weapons against South Africa's native population, as well as in conflicts with Mozambique
1991	During the Persian Gulf War, the Biological Weapons Convention was broken
1995	Aum Shinrikyo Cult released a nerve agent in Tokyo subway stations. They killed 12 people and injured several others. This cult has attempted many other times to do this again
2001	Anthrax was sent through the U.S. Mail and five people died and seventeen people were severely injured

According to the Centre for Disease Control and Prevention in the USA (CDC), bacterial agents are classified based on the overall criteria and weighting in one of three priority categories: A, B, or C. Agents in Category A (*Bacillus anthracis*, *Clostridium botulinum*, *Yersinia pestis*, Variola major,

Francisella tularensis, Filoviruses and Arenaviruses) can cause the greatest harm with mass casualties, and most require broad-based public health efforts. Category A agents also have a moderate to high level of contagiousness or a heightened general public awareness that could cause mass public fear and civil disruption. Most Category B agents (*Brucella* species, *Clostridium perfringens*, *Salmonella* species, *Escherichia coli* O157:H7, *Shigella*, *Burkholderia mallei*, *Burkholderia pseudomallei*, *Chlamydia psittaci*, *Coxiella burnetii*, toxin from *Ricinus communis*, *Staphylococcal enterotoxin B*, *Rickettsia prowazekii*, Alphaviruses, *Vibrio cholerae*, *Cryptosporidium parvum*) generally cause less illness and death than Category A and therefore would be expected to have lower medical and public health impact. These agents also have lower general public awareness than Category A agents and require fewer special public health preparedness efforts. Biological agents that are currently not believed to present a high bioterrorism risk but which could appear as future threats are placed in Category C [211–213].

In the work reported in this PhD thesis, the study of four biological agents is reported, with further possibility to expand the spectra of targets [214].

Identification of biological agents generally involves initial recognition of bacteria (vegetative cells and spores), viruses or toxins. Nucleic acid and immuno-based methods for the identification of bacteria have been widely used in testing of food, clinical and environmental samples. These detection methods should follow several essential criteria: 1) the identification method should be target-specific; 2) an assay should be sufficiently robust to work on real samples; 3) the system should work at the point of need [109]. Due to the fact that the majority of

the methods developed only meet the first criteria, there is an urgent need for a robust, easy to use system that can be deployed *in situ* for the detection of biological agents.

Bacillus thurigiensis is a surrogate of *Bacillus anthracis* (gram-positive, rod-shaped bacteria), which causes Anthrax. Anthrax can be found naturally in soil and commonly affects domestic and wild animals around the world. People can get sick with anthrax if they come in contact with infected animals or contaminated animal products. Contact with anthrax can cause severe illness in both humans and animals [215]. There are four forms of Anthrax: cutaneous (most common form), inhalation (most deadly form), gastrointestinal and injectable forms (very rare). PCR has been widely used to characterise *B. thurigiensis* [216,217] as well as multiplexed real time PCR assays [218,219].

Francisella tullarensis is a gram-negative coccobacillary or rod-shaped bacteria that causes Tularemia [220]. *F. tularensis* is found in widely diverse animal hosts and habitats and can be recovered from contaminated water, soil, and vegetation. Epizootic infection with sometimes extensive deaths of animal hosts may herald outbreaks of tularemia in humans [221]. *F. tularensis* has been identified using cultivation and molecular techniques including PCR [222,223] and real time PCR [224–227]. The most widely used serological analysis technique is the detection of specific antibodies in serum. Immunological assays like ELISA and Western blot also can be used for detection of tularemia [228]. Recently, several isothermal detection assays were developed to detect the pathogen alone [129,176,229] and in a multiplexed format [130].

Yersinia pestis is a gram-negative, rod-shaped coccobacillus, transmitted by the Oriental rat flea *Xenopsylla cheopis*, and causes Plague.

It can infect both animals and humans [230]. Plague has three main forms: pneumonic, septicemic, and bubonic [231]. All three forms have been responsible for a number of high-mortality epidemics throughout human history [230]. The "gold standard" for plague detection is bacteriological isolation of *Y. pestis*. Specific bacteriophage and biochemical tests are used to identify the bacteria (inoculation of laboratory animals, growth in special culture media) [232]. Conventional PCR [233,234] and real-time PCR assays [235–237] are also widely used for plague detection. Lateral flow [238] and immunoassays are other approaches as well as several RPA detection assays [130,175].

Brucella melitensis is a gram-negative coccobacillus bacterium from the *Brucellaceae* family that causes Brucellosis. This zoonotic bacteria generates serious, debilitating and sometimes chronic diseases that can affect a variety of organs [239]. Diagnosis of human Brucellosis is still based upon the isolation of *Brucella* sp. from the blood or other clinical samples of infected patients [240]. Alternatively, serological methods are used when cultures remain negative [241] and, among these, the Wright test is still considered the standard method [242]. However, low specificity and cross-reactions are the major limitation of serological techniques. Thus, in recent years, methods of molecular biology have been used increasingly often in the diagnostics of brucellosis, particularly PCR [243–245] and real-time PCR [246–249].

1.5.2. Toxic microalgae

Recently, there has been an increase in the number of poisonings related to consumption of seafood contaminated by toxic algae [250], caused by biological toxins produced from marine and freshwater microalgae. One of the more dangerous features of algae (*e.g.* Dinoflagellates) is their ability to migrate in order to find optimal conditions of temperature and

nutrients for their growth, achieving speeds of even faster than 10 m/day. These algal species have the potential to become dominant and produce harmful algal blooms [251].

Karlodinium is one of the toxic Dinoflagellate species, producing Karlotoxins, whose toxic activity is related to the disruption of the cell membrane by specific binding to cholesterol and creation of pores in the membrane [252]. The most widely spread species *Karlodinium veneficum* is one of the most problematic and well-studied toxic bloom-forming microalgae known [253–256] and its ichtyotoxicity was already demonstrated in the 1950s [257]. Recently, a new Karlodinium species, *K. armiger*, was isolated in the Mediterranean Sea, and has been associated with several reported fish kills and is therefore considered to be an ichthyotoxic species [253,258,259]. Due to the fact that detection of *K. armiger* is less studied, we chose it as a model system for the development of a RPA amplification and detection system. Identification of microalgae in generally done using traditional, morphology-based methods: light microscopy, SEM, TEM [258]. These methods require detailed knowledge of the species diversity and are extremely time consuming and not always accurate. Several alternative Dinoflagellate detection methods based on molecular assays have been developed in the last decade [260]. These methods include restriction fragment length polymorhism [261], heteroduplex mobility assay [262], PCR [258] and real-time PCR [263,264], fluorescent *in situ* hybridisation [265], nucleic acid based sequence amplification [266] and sandwich hybridisation assay [267]. More recently, detection methods for some of the most common toxic Dinoflagellates were developed based on bead or nanoparticle array technology [268].

1.6. Objectives

Current efforts in the development of biosensors focus on new platforms for accurate and sensitive analysis for molecular diagnostics. With the current general availability of dangerous microorganisms in large quantities, there is a fear about the possibility of using biological agents for bioterrorism attacks and late detection and diagnosis could lead to a high level of infection or massive deaths. On the other hand, harmful influence from the outside is not the only danger for humans. Simple consumption of toxic microalgae contaminated seafood may result in similar consequences. At this moment, many of the commercial tests available are slow, expensive, require large amounts of sample material, and can lead to false positive or negative results. Considering this, an electrochemical biosensor is an attractive alternative for the early detection of these pathogenic species and the early diagnosis of associated diseases. To achieve this goal, specific objectives for the biosensor development have been set:

- Establish the optimal surface functionalisation approach in order to achieve high sensitivity, stability and minimisation of non-specific interactions
- Establish the optimal hybridisation and detection conditions
- Study cross-reactivity and possibility of multiplex measurements

However, not only the biosensor platform has a significant importance. Most molecular diagnostic techniques require amplification of DNA because of initially low analyte quantities. Thermal amplification requires thermal cycling and is inherently laboratory based. To overcome this limitation, several isothermal techniques stepped have been

reported. One of them is recombinase polymerase amplification (RPA), which facilitates rapid (5-10 minutes) DNA amplification at a constant isothermal (37 – 42 °C) temperature.

Exploiting the advantages of RPA, the main objective of this doctoral thesis is to develop isothermal amplification protocols compatible with a portable, easy-to use device that can be deployed for analysis of genetic material present in biological agents or toxic microalgae at the point-of-care/need. (*Fig. 1.11*).

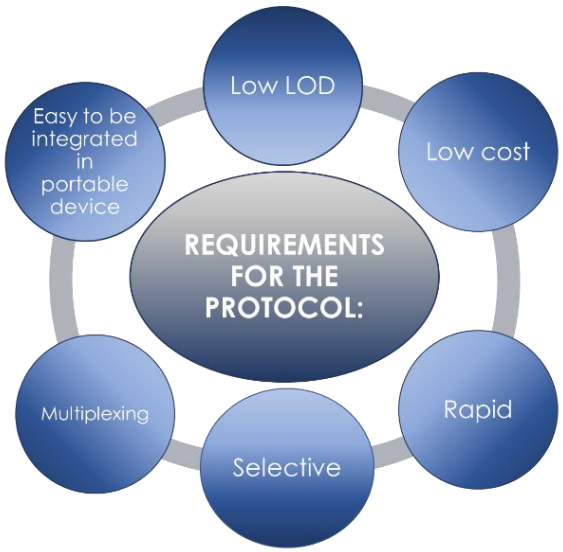


Figure 1.11 Requirements for the developed protocol for target detection

In order to achieve the overall objective of the PhD, several sub objectives were achieved, including

- Demonstration of liquid-phase singleplex and multiplex isothermal recombinase polymerase amplification
- Demonstration of solid-phase isothermal amplification of synthetic and genomic DNA on microtitre plates (optical detection) and electrodes (electrochemical detection)

- Preparation of redox-labelled dNTPs and their incorporation in DNA amplified using thermal cycling and PCR and isothermal RPA

This PhD work will contribute in different areas of molecular diagnostics as it combines a concept of isothermal amplification with electrochemical quantitative and qualitative sensing platform method for a simple detection system with further possible incorporation in a portable device for use at the point-of-need.

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CHAPTER 2

Isothermal multiplex amplification system for detection of biological agents

UNIVERSITAT ROVIRA I VIRGILI
DEVELOPMENT OF DIAGNOSTIC PLATFORM FOR DETECTION OF BIOLOGICAL AGENTS AND TOXIC MICROALGAE USING ISOTHERMAL AMPLIFICATION.
Olena Mayboroda

Isothermal multiplex amplification system for detection of biological agents

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Keywords Recombinase Polymerase Amplification, *Bacillus thuringiensis*, *Francisella tularensis*, *Yersinia pestis*, *Brucella melitensis*, multiplex "one-pot" detection

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2.1. Abstract

Nucleic acid amplification techniques (NAAT) are used in the field of molecular diagnostics for the detection and analysis of specific sequences, and routinely use the polymerase chain reaction. However, for deployment of NAATs at the point-of-need, PCR has a range of limitations including the need for a power source / electricity, instrumentation and skilled personnel. To address these drawbacks, several approaches for isothermal amplification have been developed, with the recombinase polymerase amplification (RPA) being particularly suitable as it operates at a stable isothermal temperature (37 – 42 °C), without an initial DNA melting step and amplification can be achieved in just 5-10 min. In this work, RPA was used for the simultaneous amplification of four biological agents using gel electrophoresis for detection of the obtained amplicons. The overall amplification time was less than 30 min at a constant temperature of 40 °C. The developed assay showed high specificity with no cross-reactivity between non-specific targets and primer sets.

2.2. Introduction

The recent increase of terrorism threats and the risk associated with the use of various microorganisms as biological weapons requires urgent attention to develop an easy, rapid, affordable and reliable system for their detection. Advances in biotechnology, biochemistry and bioengineering areas and increased ease of access have facilitated the development and production of biological agents. Even though the use of biological weapons is prohibited under international humanitarian law [1], as well as by a variety of international treaties, the simplicity in

deployment and and ease in obtaining these biological agents could lead to further spread of biological weapons [2].

The Centre for Disease Control and Prevention (CDC) classified the bacterial agents based on the overall criteria and weighting in one of three priority categories: A, B, or C. Agents in Category A (*Bacillus anthracis*, *Clostridium botulinum*, *Yersinia pestis*, Variola major, *Francisella tularensis*, Filoviruses and Arenaviruses) cause the greatest harm with mass casualties. Most Category B agents (*Brucella* species, *Clostridium perfringens*, *Salmonella* species, *Escherichia coli* O157:H7, *etc*) generally cause less illness and death. Biological agents that are currently not believed to present a high bioterrorism risk, but which could appear as future threats are placed in Category C [3–5].

In this work we focused our study on the simultaneous detection of four biological agents: *Category A* **1** - *Bacillus thurigiensis* as a surrogate of *Bacillus anthracis* (gram-positive, rod-shaped bacteria), that causes four possible forms of Anthrax: cutaneous (most common form), inhalation (most deadly form), gastointestinal and injection forms (very rare) [6]; **2** - *Francisella tullarensis* (gram-negative coccobacillary or rod-shaped bacteria) that causes Tularemia [7], an epizootic infection sometimes resulting in extensive deaths of animal hosts, which may also herald outbreaks of tularemia in humans [8]; **3** - *Yersinia pestis* (gram-negative, rod-shaped coccobacillus) causes Plague, which can infect both animals and humans, in three main forms: pneumonic, septicemic, and bubonic plagues [9]. All three forms were responsible for a number of high-mortality epidemics throughout human history [10]; *Category B* **4** - *Brucella melitensis* (gram-negative coccobacillus bacterium from the *Brucellaceae*

family) that causes Brucellosis, a serious, debilitating and sometimes chronic diseases that can affect a variety of organs [11].

In order to prevent or minimise the damage from any intentional release of biological agents, time effective detection or diagnosis is critical [12]. The development of the Polymerase Chain Reaction in 1983 [13] revolutionised the field of molecular diagnostics, allowing the amplification and specific detection of ng quantities of DNA. However, PCR suffers from several drawbacks such as the need for thermal cycling that requires laboratory based instrumentation, as well as a risk of sample contamination, cross-reactivity, false-positive results and sample pre-treatment to avoid problem with inhibitors that can be found in complex matrices such as blood, urine and saliva [14,15].

In recent years, alternative DNA amplification methods, based on isothermal amplification techniques, such as loop mediated isothermal amplification (LAMP), nucleic acid sequence based amplification (NASBA), helicase-dependent amplification (HDA), strand displacement amplification (SDA), rolling circle amplification (RCA) and recombinase polymerase amplification (RPA) have been developed. Most of them aim to overcome the limitations of PCR using various accessory proteins to avoid thermal cycling[16,17]. In the work reported herein, RPA was the method of choice, as it operates at a constant temperature of 37 - 42 °C, without an initial melting step, and achieving amplification levels similar to PCR in as little as 5–10 minutes [18]. There have been several reports of the amplification of biological agents using RPA and their subsequent detection including *Bacillus* with an LOD of only 16 copies in 10 min [19]; *Francisella* with an LOD of 19 copies in 10 min [19–23]; *Yersinia* with an LOD

starting of 16 copies in 8 min [19,24] and *Brucella* with an LOD of just 3 copies in 20 min [25].

Whilst the majority of reports detailing the use of RPA are based on singleplex assays, multiplexed amplification and detection is of increasing importance, not only for the detection of biological agents but also in areas of food quality control, personalised medicine and environmental monitoring. To date, several RPA multiplex assays have been reported for the detection of several targets using various detection systems. [18,26] However, not all of the reported systems are truly multiplex, and some of the developed methods are in reality parallelised singe reaction assays. [27] The purpose of the present work was to develop a "one-pot" multiplexed RPA assay for the simultaneous amplification of four biological agents.

2.3. Materials and methods

2.3.1. Chemicals

RPA kit TwistAmp Basic was obtained from TwistDx Ltd. (Babraham, UK). GelRedTM Nucleic Acid Gel Stain (Biotium, Barcelona, Spain) and low-range ultra-agarose gel powder was purchased from Bio Rad Laboratories S.A. (Barcelona, Spain). PureLink Quick Gel Extraction and PCR Purification Combo Kit were obtained from Invitrogen Corporation (Barcelona, Spain). All other chemicals were purchased from Sigma-Aldrich S.A. (Barcelona, Spain) and used as received.

2.3.2. DNA sequences

Synthetic oligonucleotides were obtained as lyophilised powder from Biomers.net (Ulm, Germany).

B. thuringiensis forward primers (FwP) (23-mer):

5'-ATGGCTTCTCCTGTAGGGTTTTC-3'

B. thuringiensis reverse primers (RvP) (21-mer):

5'-GCTGCATTTCCCATGGTTCCA-3'

Template *B. thuringiensis* DNA (116 bp):

5'AGGGCATCAAATAATGGCTTCTCCTGTCGGTTTTTCGGGGCCAGAATTCA CGTTTCCGCTATATGGAACCATGGGAAATGCAGCTCCACAACAACGTATTG TTGCTCAACTAGGTC-3'

F. tularensis forward primers (FwP) (21-mer):

5'-ATTACAATGGCAGGCTCCAGA-3'

F. tularensis reverse primers (RvP) (23-mer):

5'-TGCCCAAGTTTTATCGTTCTTCT-3'

Template *F. tularensis* DNA (116 bp):

5'AAGGAAGTGTAAGATTACAATGGCAGGCTCCAGAAGGTTCTAAGTGCCAT GATACAAGCTTCCCAATTACTAAGTATGCTGAGAAGAACGATAAAACTTGG GCAACTGTAACAGTT-3'

Y. pestis forward primers (FwP) (23-mer):

5'-GTAATAGGTTATAACCAGCGCTT-3'

Y. pestis reverse primers (RvP) (31-mer):

5'-ATCATGTGCCCGAACCCAGTCGCTGAATTTA-3'

Template *Y. pestis* DNA (132 bp):

5'GTGCGGGTAATAGGTTATAACCAGCGCTTTTCTATGCCATATATTGGACTTG CAGGCCAGTATCGCATTAATGATTTTGAGTTAAATGCATTATTTAAATTCAGC GACTGGGTTCGGGCACATGATAATGAT-3'

B. melitensis forward primers (FwP) (22-mer):

5'-GCTCGGTTGCCAATATCAATGC-3'

B. melitensis reverse primers (RvP) (20-mer):

5'-GGGTAAAGCGTCGCCAGAAG-3'

Template *B. melitensis* (151 bp):

5'GCTCGGTTGCCAATATCAATGCGATCAAGTCGGGCGCTCTGGAGTCCGGCT
TTACGCAGTCAGACGTTGCCTATTGGGCCTATAACGGCACCGGCCTTTATGAT
GGCAAGGGCAAGGTGGAAGATTTGCGCCTTCTGGCGACGCTTTACCC3'

DNA was dissolved in high purity deionised water (18 MΩ) produced with a Milli-Q RG system (Millipore Ibérica, Spain), aliquoted and diluted to the appropriate concentrations using Milli-Q water and used without further purification.

2.3.3. PCR protocol

PCR was performed in a final volume of 50 µL volume using 1 x Master mix (Invitrogen Corporation, Barcelona, Spain), 200 nM primers, 5 mM $MgCl_2$, 200 µM dNTPs, 0.5 % w/v Bovine serum albumin (BSA) (Hersteller, Ort, Land), template DNA, Milli-Q water and *Tfi* DNA polymerase. For the non-template control, Milli-Q water was used instead of specific DNA. PCR was performed using a Peltier Thermal Cycler (Bio Rad Laboratories S.A., Barcelona, Spain) with the following program: initial denaturation at 95 ^{o}C for 2 minutes, followed by 15 cycles of denaturation at 95 ^{o}C for 30 seconds, annealing at 65 ^{o}C for 30 seconds and elongation at 72 ^{o}C for 30 seconds. Following the last cycle, elongation was continued for 5 minutes.

For multiplex PCR experiments, all four sets of primers, each of 200 nM concentration, and four targets were mixed in the same tube in order to amplify four targets in a ”one-pot” reaction. The amplification

protocol consisted of 15 cycles using the same thermal cycling conditions as used for singleplex. Successful amplification was verified via electrophoresis using 12.5 % w/v polyacrylamide gel in 1 x Tris/Borate/EDTA buffer (TBE) for 60 min at 100 V. PCR dsDNA amplicons were purified according to the instructions provided with the PureLink PCR Purification Combo Kit. The purified amplicon concentration was determined by UV-Visible absorption spectrophotometry at λ=260 nm using a UV-Visible Spectrophotometer Cary 100 Bio (Varian Iberica, S.L., Barcelona, Spain).

2.3.4. Recombinase Polymerase Amplification (RPA) protocol

RPA was performed as specified in the protocol provided by TwistDx (*Fig. 2.1*). The singleplex reaction was carried out in a 50 µL volume using the TwistAmp Basic kit, 240 nM PCR primers, DNA template, 14 mM magnesium acetate and TwistAmp rehydration buffer. All reagents, with the exception of the template DNA and Mg-acetate were prepared in the master mix provided by TwistDx, which was distributed into 0.2 mL reaction tubes.

For multiplex RPA experiments, all four sets of primers with different concentrations (*Table 2.1*) were mixed in the same tube in order to amplify the four targets in the same reaction. For both singleplex and multiplex reactions, magnesium acetate was pipetted into the tube lids. Subsequently, one or all four template DNA were added into the tubes. The lids were closed and magnesium acetate centrifuged into the tubes to start the reaction. Subsequently, the tubes were vigorously inverted 8-10 times to mix and again centrifuged. The RPA mixture was placed in an

incubator block at 40 °C and incubated for 4 minutes, and then inverted 8-10 times to mix, again centrifuged and placed back in the incubator block, for a further 20 minutes. Amplification was again verified using 12.5 % w/v polyacrylamide gel via electrophoresis in 1 x TBE buffer at constant voltage of 100 V for 60 minutes.

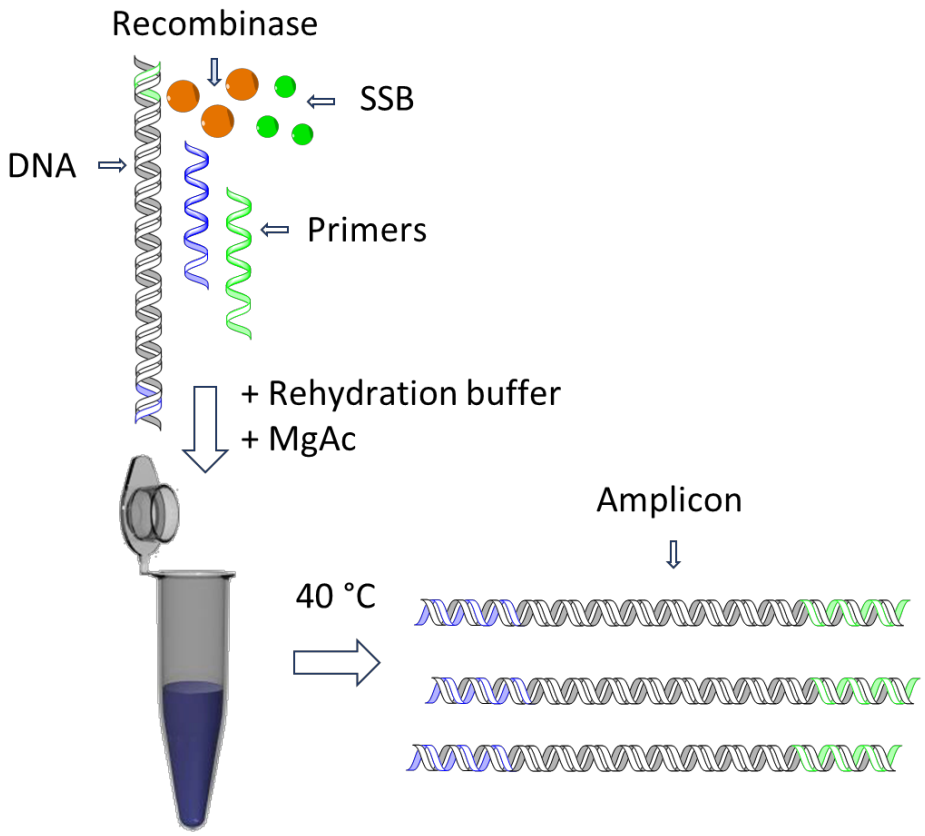

Figure 2.1 Schematic representation of homogeneous liquid-phase RPA

Even though only two primers are needed for a successful singleplex RPA amplification whereas LAMP typically uses six primers, an optimisation of conditions is still required for multiplex RPA. Various primer concentrations were evaluated and optimised for the multiplex

RPA. The starting point was the primer concentration proposed by the RPA kit manufacturer – 500 nM. Since this amount of primers gave a lot of background amplification, several lower concentrations were tested: 400, 320, 250, 240, 200, 160, 150, 100 and finally 80 nM. We noticed that the choice of primer concentration depends highly on the sequence of both the target DNA and the primers themselves and we thus used concentrations optimal for each target separately and this approach gave the possibility to achieve "one pot" multiplexing. The temperature and the duration of amplification also play an important role. Due to the fact that the RPA works optimally in the range of 37 – 42 °C, we tested different amplification temperatures starting from 35 °C and going up to 37, 40 and 45 °C to see which temperature gives the best level of amplification. Different amplification times were also evaluated going from 15 minutes to 25, 35, 45 and 60 minutes. The optimal primer concentrations are presented in Table 2.1, and a temperature of 40 °C and amplification for 25 minutes allowed amplification of all four of the chosen targets in a "one-pot" reaction with easy gel electrophoresis visualisation due to the size difference of the amplicons (Fig. 2.4).

Table 2.1 Primer concentrations for RPA multiplex amplification

Target	*Primer concentration (nM)*
Bacillus thuringiensis	240
Francisella tularensis	320
Yersinia pestis	80
Brucella melitensis	160

2.4. Results and discussion

DNA was firstly amplified using conventional PCR to test the efficiency of the primers designed. Figure 2.2 lanes 1, 3, 5, 7 shows amplicons obtained for singleplex amplification of synthetic DNA. No non-specific amplification was observed (Fig. 2.2 lanes 2, 4, 6, 8). Successful "one-pot" multiple PCR amplification of four targets is shown in Figure 2.2 lane 9, 10.

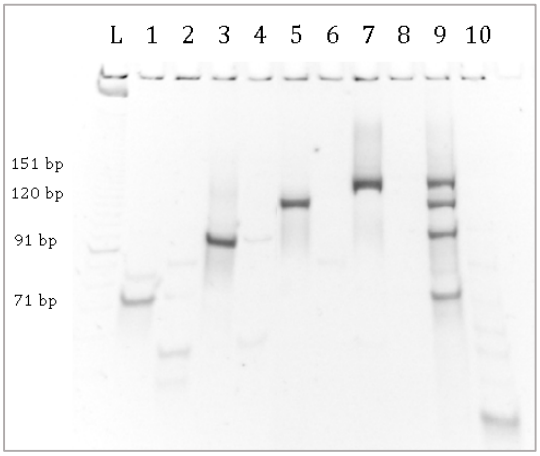

Figure 2.2 Electrophoresis of PCR singleplex and multiplex amplicons in 12.5 %. w/v polyacrylamide gel L – 10 bp ladder; **1** – PCR positive control *B. thuringiensis* (71 bp); **2** – PCR negative control *B. thuringiensis*; **3** – PCR positive control *F. tularensis* (91 bp); **4** – PCR negative control *F. tularensis*; **5** – PCR positive control *Y. pestis* (120 bp); **6** – PCR negative control *Y. pestis*; **7** – PCR positive control B. melitensis (151 bp); **8** – PCR negative control B. melitensis; **9** – PCR multiplex positive control *B. thuringiensis* (71 bp), *F. tularensis* (91 bp), *Y. pestis* (120 bp) and *B. melitensis* (151 bp); **10** – PCR multiplex negative control.

RPA was carried out using a modified protocol with conventional PCR primers, facilitating a simplification in their design, as shorter primers are of lower cost and are less prone to primer-dimer formation. RPA singleplex amplicons for synthetic DNA can be seen in Figure 2.3 Lanes 1, 3, 5, 7). No non-specific amplification was observed (Fig. 2.3

Lanes 2, 4, 6, 8). The multiplexed amplification of four targets can be seen in Figure 2.4 Lanes 1 and 2.

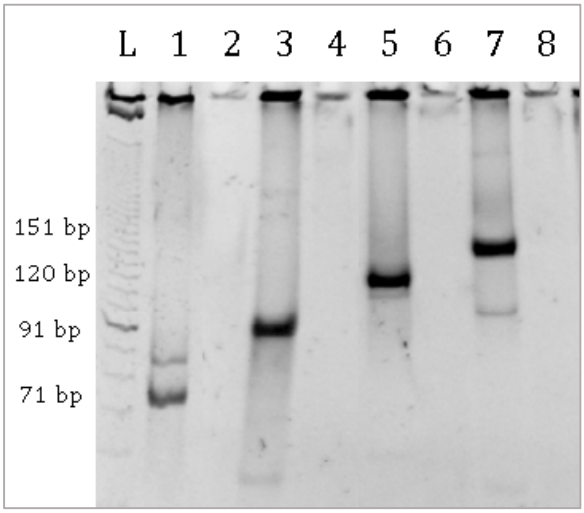

Figure 2.3 Electrophoresis of RPA singleplex amplicons in 12.5 % w/v polyacrylamide gel. L – 10 bp ladder; **1** – RPA positive control *B. thuringiensis* (71 bp); **2** – RPA negative control *B. thuringiensis*; **3** – RPA positive control *F. tularensis* (91 bp); **4** – RPA negative control F. tularensis; **5** – RPA positive control *Y. pestis* (120 bp); **6** – RPA negative control *Y. pestis*; **7** – RPA positive control *B. melitensis* (151 bp); **8** – RPA negative control *B. melitensis*.

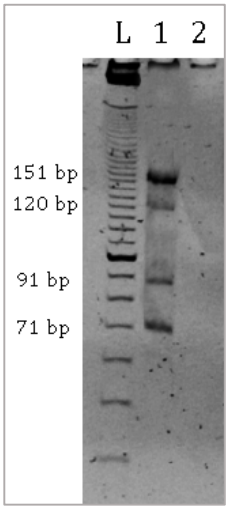

Figure 2.4 Electrophoresis of RPA multiplex amplicons in polyacrylamide gel 12.5 % in homogeneous amplification. L – 10 bp ladder; **1** – RPA positive control *B. thuringiensis* (71 bp), *F. tularensis* (91 bp), *Y. pestis* (120 bp) and *B. melitensis* (151 bp); **2** – RPA negative control.

Cross reactivity experiments were performed for both PCR and RPA in order to confirm that primers for one target do not amplify the template of another target DNA. All primers showed no cross-reactivity.

The obtained results thus demonstrate the possibility of RPA amplification of *B. thuringiensis*, *F. tularensis*, *Y. pestis* and *B. melitensis* separately and in multiplex reaction using conventional PCR primers for synthetic DNA.

2.5. Conclusions

This study is a proof-of-concept for the multiplex amplification of four biological agents using gel electrophoresis detection system for synthetic DNA. The system has high selectivity as verified by the lack of amplification of non-specific sequences. RPA has clear advantages over

PCR for portable devices that can be used in the field and it is the focus of our future work based on the proof-of-concept demonstrated here.

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CHAPTER 3

Isothermal solid-phase amplification system for detection of *Yersinia pestis*

UNIVERSITAT ROVIRA I VIRGILI
DEVELOPMENT OF DIAGNOSTIC PLATFORM FOR DETECTION OF BIOLOGICAL AGENTS AND TOXIC MICROALGAE USING ISOTHERMAL AMPLIFICATION.
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Keywords Recombinase Polymerase Amplification, *Yersinia pestis*, solid-phase detection, ELONA

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3.1. Abstract

DNA amplification is required for most molecular diagnostic applications, but conventional polymerase chain reaction (PCR) has disadvantages for field testing. Isothermal amplification techniques are being developed to respond to this problem. One of them is the recombinase polymerase amplification (RPA) that operates at isothermal conditions without sacrificing specificity and sensitivity in easy- to-use formats. In this work, RPA was used for the optical detection of solid-phase amplification of the potential biowarfare agent *Yersinia pestis*. Thiolated forward primers were immobilised on the surface of maleimide-activated microtitre plates for the quantitative detection of synthetic and genomic DNA, with elongation occurring only in the presence of the specific template DNA and solution phase reverse primers. Quantitative detection was achieved via the use of biotinylated reverse primers and post-amplification addition of streptavidin–HRP conjugate. The overall time of amplification and detection was less than 1 h at a constant temperature of 37 °C. Single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) sequences were detected, achieving detection limits of $4.04*10^{-13}$ and $3.14*10^{-16}$ M, respectively. The system demonstrated high specificity with negligible responses to non-specific targets.

3.2. Introduction

Yersinia pestis is the cause of bubonic and pneumonic plague and was responsible for several pandemics in Europe during the Middle Ages [1]; nowadays, it occurs naturally in parts of Africa and Asia and in the western USA. The disease is transmitted by the Oriental rat flea

Xenopsylla cheopis, and it can infect both animals and humans. The World Health Organisation (WHO) reports that plague results in 30 to 60 % fatalities without prompt treatment. Furthermore, *Y. pestis* is considered as a warfare agent by the Centers for Disease Control and Prevention due to its lethality rate [2]; thus, a rapid, sensitive and easy-to-use assay for *Y. pestis* detection addresses a mature healthcare and social need.

The "gold standard" for plague detection is bacteriological isolation of *Y. pestis*. Specific bacteriophage and biochemical tests are used to identify the bacteria (inoculation of laboratory animals, growth in special culture media). These methods are both time consuming and expensive. Moreover, incorrect procedures may result in damage to bacteria, including dehydration, contamination of the samples or death of the bacteria, leading to false-positive or false-negative results as a consequence of the impossibility of cell division and growth in agar media [3].

Various assays have been developed to overcome these problems. Conventional polymerase chain reaction (PCR) provides a limit of detection (LOD) of 10–100 colony- forming units (CFU) within 4 h [4] but suffers from typical drawbacks of amplification techniques such as the need to heat to 95 °C, as well as a high risk of sample contamination, cross-reactivity and false-positive results [4,5]. Recently, several real-time PCR assays have been developed for the direct detection of *Y. pestis* in a shorter timeframe, having less cross- contamination and better specificity as they use fluorescent hybridisation probes, facilitating real-time detection [6–8]. Whilst these techniques are highly specific and sensitive, they are not always suitable for application in a portable device.

To address this caveat, several lateral flow assays have been developed. One of them was reported by Yan et al. [9] in 2006 to detect *Y. pestis* using up-converting phosphor technology. Immunoassays are another approach that aims to help in development of a rapid, easy and specific detection system. But most of them are antibody based that limits their wide use [10,11]. Some of these technologies are nowadays commercially available, such as theSMART II *Yersinia pestis* Anti-F1 detection kit (New Horizons Diagnostics Corporation), Plague Bio Threat Alert (Tetracore) and BADD Plague (ADVNT) as well as immunofiltration assays including the ABICAP classic test kit—*Y. pestis* (Senova GmbH). However, these devices permit detection but not amplification, an example of a portable device capable of detection being the recently reported PicoReal device, which weighs 11 kg [12]. The immunofiltration assays have high sensitivity, with a limit of detection (LOD) of 104 bacteria per millilitre, but they show limited specificity and have a complicated procedure that is incompatible with field testing, whilst the available rapid and easy-to-use lateral flow assays show very limited sensitivity [9–12].

Recently, alternative DNA amplification methods, based on isothermal amplification techniques, have been developed. These techniques provide a significant advantage over conventional methodologies as most of them do not require thermal cycling and operate at low and constant temperatures. In the work reported here, we exploited the recombinase polymerase amplification (RPA), which could meet all requirements of lateral flow devices, as it can operate at a constant temperature of 37 °C without an initial melting step with amplification equivalent to PCR being achieved within 5–10 min [13]. To

date, this technique has successfully been exploited in commercially available tests for *Salmonella enterica* INVA gene using real-time fluorescent detection (TwistGlow Salmonella) and lateral flow detection (TwistFlow Salmonella). These tests demonstrate PCR-like sensitivity and detection within 10–20 min, depending on the system used. In 2013, RPA and the real-time recombinase polymerase amplification (RT-RPA) fluorescent-labelled probe system for *Y. pestis* were reported to detect 16–21 DNA copies in less than 10 min at a constant temperature of 42 °C [14], and label-free, multiplex RT-RPA of three bacterial pathogens with LODs of 10–100 CFU has also been reported [15]. Santiago-Felipe et al. [16] reported the RPA assay for two bacterial species using a microarray format on a DVD surface, achieving a LOD of 10^1–10^2 CFU per millilitre. However, the requirement for initial dehybridization, sample amplification in the tube before detection and usage of antibodies still limits the implementation of the system in field testing [16].

The purpose of the work reported here was to develop a method of *Y. pestis* detection to be subsequently exploited in integrated (amplification and detection) portable devices. RPA for DNA amplification was exploited and transferred from a homogenous to a heterogenous format using enzyme-linked oligonucleotide assay (ELONA) for the detection of single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA). Synthetic DNA was used for method development whilst genomic DNA from real samples was used as target for the proof of concept (*Fig. 3.1*).

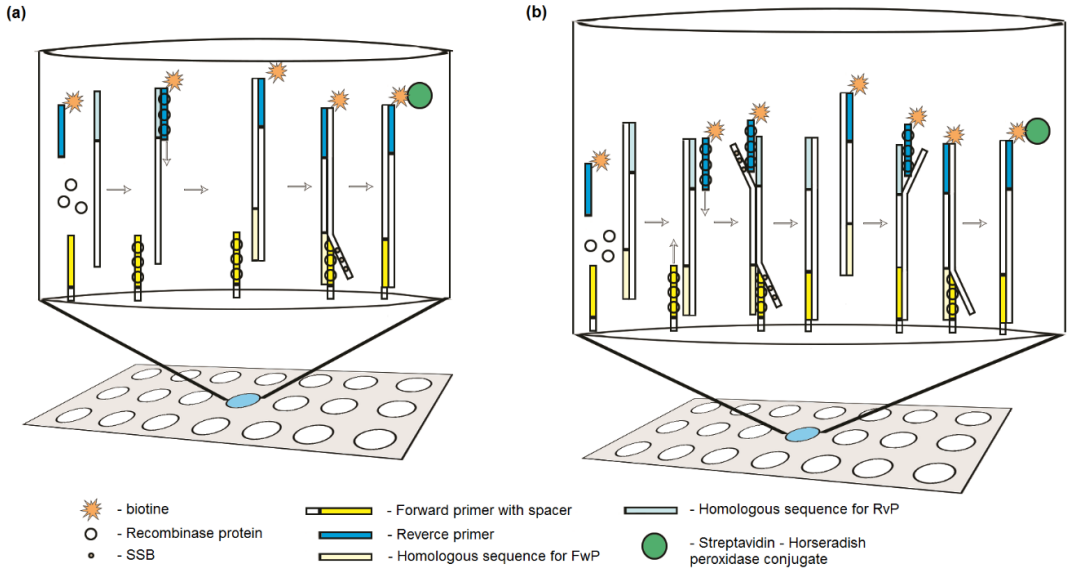

Figure 3.1 Schematic of solid-phase RPA on Maleimide activated microtitre plate. **a** Amplification using ssDNA and **b** Amplification using dsDNA as a template

3.3. Materials and methods

3.3.1. Chemicals

RPA kit TwistAmp Basic was obtained from TwistDx Ltd. (Babraham, UK). GelRedTM Nucleic Acid Gel Stain (Biotium, Barcelona, Spain) and low-range ultra-agarose gel powder was from Bio Rad Laboratories S.A. (Barcelona, Spain). PureLink Quick Gel Extraction and PCR Purification Combo Kit were obtained from Invitrogen Corporation (Barcelona, Spain). Maleimide-activated microtitre plates were obtained from ThermoScientific (Madrid, Spain). Streptavidin–peroxidase from *Streptomyces avidinii* and peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate system for ELISA were purchased from Sigma-Aldrich S.A. (Barcelona, Spain). All other

chemicals were purchased from Sigma-Aldrich S.A. (Barcelona, Spain) and used as received.

3.3.2. DNA sequences

Synthetic oligonucleotides were obtained as lyophilised powder from Biomers.net (Ulm, Germany).

Y. pestis forward primers (FwP) (23-mer):

5'-GTAATAGGTTATAACCAGCGCTT-3'

5'-SH-$(CH_2)_6$-15T-GTAATAGGTTATAACCAGCGCTT-3'

Y. pestis reverse primers (RvP) (31-mer):

5'-ATCATGTGCCCGAACCCAGTCGCTGAATTTA-3'

5'-biotin-ATCATGTGCCCGAACCCAGTCGCTGAATTTA-3'

CDH1 (non-specific forward primer) (20-mer)

5'-SH-$(CH_2)_{24}$-15T-CCGATTACGAACCAGCCTAT-3'

Template *Y. pestis* DNA (132 bp):

5'GTGCGGGTAATAGGTTATAACCAGCGCTTTTCTATGCCATATATTGGACTTG
CAGGCCAGTATCGCATTAATGATTTTGAGTTAAATGCATTATTTAAATTCAGC
GACTGGGTTCGGGCACATGATAATGAT-3'

Template *Francisella tularensis* DNA (non-complementary target) (134 bp):

5'TGACCGGCAGCAAAATGTAAGGAAGTGTAAGATTACAATGGCAGGCTCCAG
AAGGTTCTAAGTGCCATGATACAAGCTTCCCAATTACTAAGTATGCTGAGAAG
AACGATAAAACTTGGGCAACTGTAACAGTT-3'

DNA was dissolved in high purity deionised water (18 MΩ) produced with a Milli-Q RG system (Millipore Ibérica, Spain), aliquoted and diluted to the appropriate concentrations using Milli-Q water and used without further purification.

Y. pestis real samples were kindly provided from the Institute of Bacterial Infections and Zoonoses, Friedrich-Loeffler-Institute. Briefly, the DNA extraction and purification protocol consisted of the preparation of a *Y. pestis* bacteria suspension using a High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany). Two hundred µL bacteria suspension including colony material harvested with 1 x phosphate-buffer saline (PBS) were prepared according to the manufacturer's instructions for isolation of total nucleic acids from cell cultures.

3.3.3. PCR protocol

PCR was performed in a final volume of 25 µL volume using 1 x Master mix (Invitrogen Corporation, Barcelona, Spain), 200 nM primers, 5 mM $MgCl_2$, 200 µM dNTPs, 0.5 % w/v Bovine serum albumin (BSA) (Hersteller, Ort, Land), template DNA (synthetic or genomic), Milli-Q water and *Tfi* DNA polymerase. For the non-specific control, Milli-Q water was used instead of specific DNA. PCR was performed using a Peltier Thermal Cycler (Bio Rad Laboratories S.A., Barcelona, Spain) with the following amplification program: initial denaturation at 95 ^{o}C for 2 minutes, followed by 26 cycles of denaturation at 95 ^{o}C for 30 seconds, annealing at 60 ^{o}C for 30 seconds and primer extension at 72 ^{o}C for 30 seconds. Following the last cycle, primer extension was continued for 5 minutes. Successful amplification was verified via electrophoresis using 2.5 % agarose gel in 1 x Tris/Borate/EDTA buffer (TBE) for 30 min at 100 V. PCR dsDNA amplicons were purified according to the protocol according to the instructions provided with the PureLink PCR Purification Combo Kit. The purified amplicon concentration was determined by UV-

Visible absorption spectrophotometry at λ=260 nm using UV-Visible Spectrophotometer Cary 100 Bio (Varian Iberica, S.L., Barcelona, Spain).

3.3.4. Recombinase Polymerase Amplification (RPA) protocol

RPA was performed as specified in the protocol provided by TwistDX. The reaction was carried out in a 50 µL volume using the TwistAmp Basic, 240 nM PCR primers, DNA template (synthetic or genomic), 14 mM magnesium acetate and TwistAmp 0.5 x rehydration buffer. All reagents, with the exception of the template DNA and Mg-acetate were prepared in a master mix provided by TwistDx with 0.25 dried enzyme pellet per reaction, which was distributed into 0.2 mL reaction tubes. Magnesium acetate was pipetted into the tube lids. Subsequently synthetic or genomic DNA was added into the tubes. The lids were closed and magnesium acetate centrifuged into the tubes to start the reaction. Subsequently, the tubes were vigorously inverted 8-10 times to mix and again centrifuged. The RPA mixture was placed in an incubator block at 40 °C and incubated for 4 minutes, and then inverted 8-10 times to mix, and again centrifuged and placed back in the incubator block, for a further 20-40 minutes. The amplification was verified using 2.5 % agarose gel via electrophoresis in 1 x TBE buffer at constant voltage 100 V for 30 minutes.

3.3.5. Detection of *Y. pestis* DNA on maleimide microtitre plates by solid phase RPA

The thiolated forward primer was immobilised on the wells of a maleimide plate by addition of 50 µL of 200 nM solution in 10 mM PBS

(pH 7.4), followed by an overnight incubation at room temperature and thorough washing with PBS containing 0.05% (v/v) Tween 20 (PBS-Tween). Blocking of any non-functionalised maleimide moities was achieved via incubation with 200 µL of 1 % casein in PBS-Tween for 1 h, and subsequent washing with PBS-Tween.

Solid phase RPA was performed in a 50 µL volume using the TwistAmp Basic, 120 nM PCR biotinylated RvPs, template DNA, 14 mM magnesium acetate, TwistAmp 0.5 x rehydration buffer and 0.25 dried enzyme pellet. All reagents with the exception of the template DNA and magnesium acetate were provided in a master mix, which was distributed into each well of the maleimide activated plate. 13.2 µL of DNA template (ssDNA or dsDNA – synthetic or genomic) and 2.5 µL Mg acetate were pipetted to initiate the reaction. The reaction took place in an incubator with a fixed temperature of 37 °C for 30 minutes. Following amplification, the plate was thoroughly washed with 200 µL of PBS-Tween 3-5 times. The amplicon was then directly labelled with 50 µL of 0.5 nM streptavidin-HRP for 15 minutes at room temperature. After the last washing with PBS-Tween, the presence of the HRP-labelled probe was observed via addition of 50 µL of TMB substrate for 2 minutes. The reaction was stopped by adding 50 µL of 1 M H_2SO_4. The absorbance was measured at 450 nm (SpectraMax 340PC384, bioNova científica s.l., Spain). In parallel, negative controls were included to demonstrate the selectivity of the assay: a) no template DNA in the RPA mix, b) non-specific DNA sequence (*F. tularensis*) and c) non-specific forward primer (CDH1). To assess the sensitivity of the assay, amplification was carried out using different starting concentrations of *Y. pestis* synthetic ssDNA (1 nM, 0.3 nM, 0.1 nM, 0.03 nM, 0.01 nM, 0.003 nM, 0.001 nM, 0.0003 nM, 0.0001 nM, 0.00003 nM,

0.00001 nM). Genomic DNA was used in a concentration of 0.1 ng/µL per reaction.

3.4. Results and discussion

DNA was firstly amplified using conventional PCR to test the efficiency of the primers designed. *Fig. 3.2 (lines b and c)* shows amplicons obtained for synthetic and genomic DNA of *Y. pestis*. No non-specific amplification was observed (*Fig. 3.2 lane h*).

The same combination of primers was tested using the RPA system. Amplification was carried out using a modified RPA protocol, using conventional PCR primers, facilitating a simplification in their design, as shorter primers are of lower cost and avoid primer-dimer formation. RPA amplicons for synthetic and genomic DNA can be seen in *Figure 3.2 (lanes d - g)*. No cross-reactivity was observed (*Fig. 3.2 lanes i and j*). The obtained results thus demonstrate the possibility of RPA amplification of *Y. pestis* using conventional PCR primers for synthetic and genomic DNA. However, homogeneous RPA results in the production of DNA amplicon by-products of random sequences. This drawback can be overcome by heterogeneous RPA due to the immobilisation of one primer on the surface. All the random amplicons remain in solution and therefore are washed away during the process avoiding the need for complex primers and additional enzymes [13].

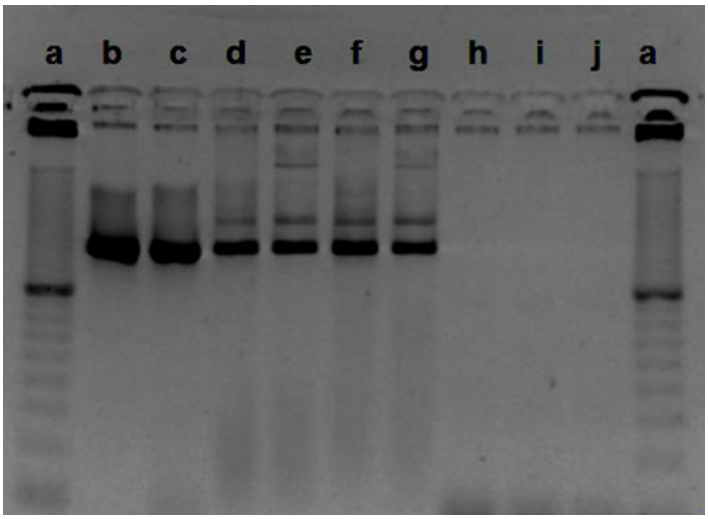

Figure 3.2 Electrophoresis of amplicons in agarose gel 2.5 % of *Yersinia pestis* DNA in homogeneous amplification. **a** – 10 bp ladder; **b** – PCR positive control synthetic; **c** – PCR positive control genomic DNA; **d** – RPA positive control synthetic 25 min; **e** – RPA positive control genom. 25 min; **f** – RPA positive control synth. 45 min; **g** – RPA positive control genomic DNA. 45 min; **h** – PCR negative control; **i** – RPA negative control 25 min; **j** – RPA negative control 45 min.

ELONA RPA on maleimide activated plates was explored as a demonstration of the heterogeneous detection system. As can be seen in *Figure 3.3*, amplification and detection of the specific target (ssDNA and dsDNA) was achieved using biotinylated reverse primers. A selectivity study using a non-specific DNA sequence (*F. tularensis*) and a non-complementary surface probe (CDH1) clearly demonstrates the ability of recombinase proteins to differentiate between target DNA and non-specific sequences in the heterogeneous assay. *Fig. 3.3a and 3.3c* show the absorbance recorded at the end of the assay for the specific amplification of *Y. pestis* ssDNA and dsDNA and the controls, using a starting DNA concentration of $1*10^{-10}$ M ($6*10^{13}$ copies per mL) for both, showing a high absorbance level for the specific amplification with low response for

the controls. *Fig. 3.3(b) and 3.3(d)* show the calibration curve obtained using different initial ssDNA and dsDNA concentrations. The linear range covers four orders of magnitude for ssDNA and five orders of magnitude for dsDNA. The limit of detection (LOD), obtained using ssDNA, was $4.04*10^{-13}$ M ($2.04*10^{11}$ copies per mL), whilst the LOD for the dsDNA was $3.14*10^{-16}$ M ($1.88*10^{8}$ copies per mL), determined as the concentration of the analyte at the mean blank signal plus three times the standard deviation of the blank.

Simon et al. [17] reported solid-phase sandwich immunoassay for *Y. pestis* with a LOD of 10^6 cells per mL, although the use of antibodies may limit the usage of this assay in in-field testing. A solid-phase RPA ELONA detection of a different warfare agent (*F. tularensis*) was recently reported by Sabate Del Rio et al. [18]. The procedure for *Y. pestis* reported here has the advantage of fewer steps in the assay, as post-amplification denaturation and subsequent hybridisation with reporter labelled probe is obviated due to the use of biotinylated RvP that inherently reduces the time needed for detection as well as the background (blank) absorbance. The LOD for ssDNA *F. tularensis* system achieved was $1.3*10^{-13}$ M, whilst the LOD of dsDNA system for *Y. pestis* achieved was two orders of magnitude lower, ($3.14*10^{-16}$ M) highlighting the power of the assay for the direct detection of dsDNA. A recent report details solid-phase optical RPA detection allowing label-free real-time amplification monitoring, with a LOD of $7.8*10^{-13}$ M for dsDNA, which is three orders of magnitude higher than that reported here [19].

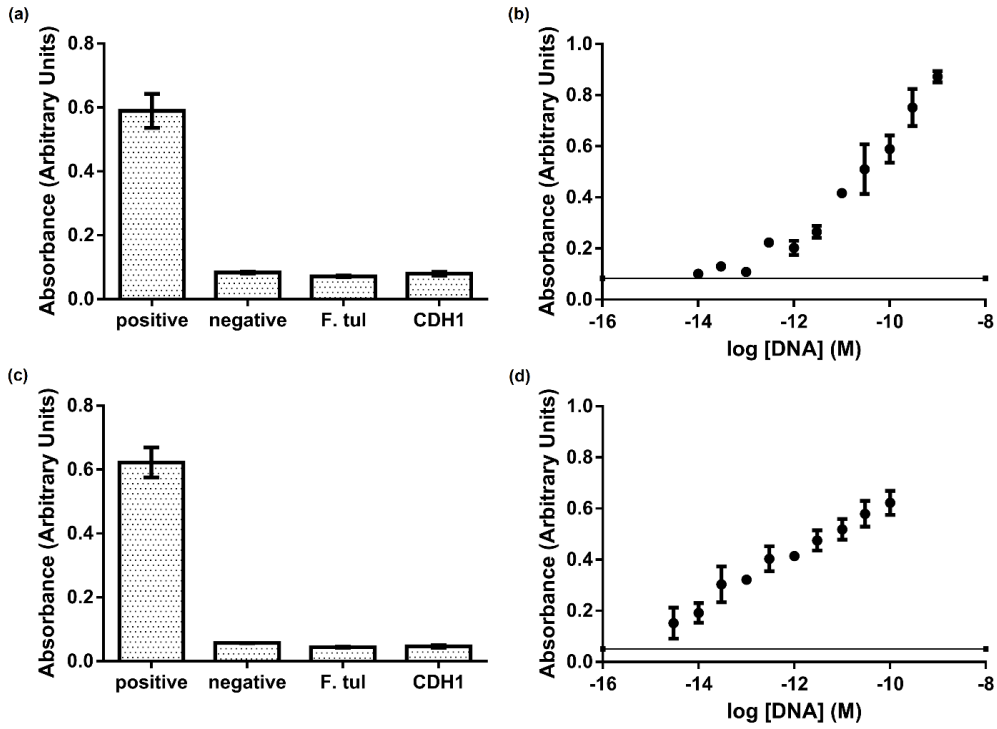

Figure 3.3 Absorbance of heterogeneous RPA assays for *Yersinia pestis* on maleimide activated plates. **(a)** Specific signal from amplicon obtained from ssDNA with controls (negative control – non-template reaction; non-specific DNA – control using non-complementary DNA *F. tularensis*; non-specific surface probe – control using non-complementary forward primer CDH1). **(b)** Calibration curve using different template concentration of ssDNA (n=3). **(c)** Specific signal from amplicon obtained from dsDNA with controls (negative control – non-template reaction; non-specific DNA – control using non-complementary DNA *F. tularensis*; non-specific surface probe – control using non-complementary forward primer CDH1). **(d)** Calibration curve using different template concentration of dsDNA (n=3)

3.5. Conclusions

This study is a proof-of-concept for the heterogeneous RPA assay for one warfare agent using optical detection system for synthetic and genomic DNA. The system has high selectivity as verified by the lack of

amplification of non-specific sequences. Homogeneous RPA has advantages over PCR for portable devices that can be used in the field because of amplification in isothermal conditions. Heterogeneous RPA provides additional advantages since there are less wash and separation steps needed and detection can be realised at the same location as amplification. This points to the possibility of a more seamless transfer of the assay into a portable, easy to use device such as a lateral flow system in the future. The usage of short PCR primers in both, homogeneous and heterogeneous RPA, it is also demonstrated. Such usage lowers the cost of the assay and simplifies its development. For practical applications a lower detection limit is necessary and the demonstration of a portable, reagentless, easy to use device is the focus of our future worked based on the proof-of-concept demonstrated here.

3.6. Acknowledgements

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CHAPTER 3

CHAPTER 4

Enhanced solid-phase recombinase polymerase amplification and electrochemical detection

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Keywords Electrochemical genosensor, solid phase recombinase polymerase amplification, surface chemistry, real samples from hares.

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4.1. Abstract

Recombinase polymerase amplification (RPA) is an elegant method for the rapid, isothermal amplification of nucleic acids. Here, we elucidate the optimal surface chemistry for rapid and efficient solid-phase RPA, which was fine-tuned in order to obtain a maximum signal-to-noise ratio, defining the optimal DNA probe density, probe-to-lateral spacer ratio (1:0, 1:1, 1:10 and 1:100), and length of a vertical spacer of the probe as well as investigating the effect of different types of lateral spacers. The use of different labelling strategies was also examined in order to reduce the number of steps required for the analysis, using biotin or horse-radish peroxidase labelled reverse primers. Optimisation of the amplification temperature used and the use of surface blocking agents was also pursued. The combination of these changes facilitated a significantly more rapid amplification and detection protocol, with a lowered limit of detection (LOD) of $1*10^{-15}$ M. The optimised protocol was applied to the detection of *Francisella tularensis* in real samples from hares and a clear correlation with PCR and qPCR results observed and the solid-phase RPA demonstrated to be capable of detecting 500 fM target DNA in real samples.

4.2. Introduction

Since the introduction of recombinase polymerase amplification [1], the system has been extensively used in a range of formats due to its' simplicity, and true isothermal nature of amplification, where no first cycle thermal denaturation step is required. Reported systems include the use of fluorescent probes for real-time amplification/detection of either DNA [2–4] or RNA [5–7], whilst other common applications include end-

point detection system using lateral flow assays [8–10]. Elegant and simple solutions using digital versatile disc (DVD) as platforms have been also reported, with the detection been carried out by a slightly modified DVD optical player [11–13].

In RPA, the need for thermal cycling used in the polymerase chain reaction is avoided and replaced by three core proteins that operate optimally between 37 °C and 40 °C. The first protein, a recombinase, binds to primers, forming filaments that can then recombine with homologous DNA in a duplex target, forcing displacement of the non-complementary strand and thus provoking the formation of a D-loop. The second protein is a single-stranded DNA binding protein, which attaches to the strand of DNA displaced by the primer, preventing the dissociation of the primer and hybridisation of the duplex target. The final core protein is a strand-displacing polymerase that copies the DNA, adding bases onto the 3' end of the primer, forcing open the DNA double helix as it progresses. When opposing primers are used, exponential amplification occurs. In solid-phase RPA [14], one of the primers is covalently linked to a surface, therefore the elongation of primers and amplification of the target, occurs in both the liquid and the solid phase simultaneously.

Solid-phase amplification has the inherent advantage that it is highly adaptable to multiplexed amplification, particularly when detection is facilitated by the surface on which amplification takes place e.g. electrodes [14], plastic slides [15], glass slides [16], ring resonators [17–19], or optical discs [11]. Additionally, the spatial separation proffered by solid-phase strategies is that detection can exploit the use of a single reporter for all the sequences, or label-free approaches, and therefore the need for different labels to differentiate between each target is avoided.

Herein we report on the improvement and simplification of an approach we previously described for the electrochemical detection of solid-phase RPA (SP-RPA), evaluating different surface chemistries and labelling strategies. In our initial report of electrochemical detection of solid-phase RPA, we used quite a convoluted system requiring post-amplification denaturation of amplified material, and subsequent hybridisation with a biotin labelled probe, followed by addition of streptavidin labelled horseradish peroxidase (SA-HRP), substrate addition and signal detection with inherent washing following each step. Therefore we evaluated the use of biotin and horseradish peroxidase (HRP) labelled primers as a means of both simplifying the system and reducing the time required. Furthermore, we explored the importance of a combination of vertical and lateral spacers to assist in the recombinase-primer complex rapidly hybridising to its' homologous sequence and initiating amplification, in an effort to reduce the duration of amplification. Finally, we applied the SP-RPA system to determine the presence of specific sequences related to *Francisella tularensis* in real samples from hares, where tularemia is most frequently observed in Europe [20].

4.3. Materials

Old formulation RPA kit TwistAmp® Basic was obtained from TwistDx Ltd. (Babraham, United Kingdom). The HRP substrate formulation tetramethylbenzidine (TMB) enhanced one component HRP membrane was purchased from Diarect AG (Germany), GelRed™ Nucleic Acid Gel Stain from Biotium (Barcelona, Spain), certified molecular biology agarose gel powder from Bio Rad Laboratories S.A. (Barcelona,

Spain) and *Thermus filiformis* DNA polymerase from Invitrogen Corporation (Barcelona, Spain). 3 mm thick polymethylmethacrylate (PMMA) was purchased from La Indústria de la Goma (Tarragona, Spain), double-sided medical grade adhesive foil from Adhesive Research (Ireland) and all other chemicals were obtained from Sigma Aldrich S.A. (Barcelona, Spain) and used as received.

Synthetic oligonucleotides designed for the detection of the pathogenic bacteria *Francisella tularensis holarctica* [21] were purchased as lyophilised powder from Biomers.net (Ulm, Germany), reconstituted at 100 μM in high purity deionised water (18 MΩ) produced with a Milli-Q RG system (Millipore Ibérica, Spain) and used without further purification (Electronic Supplementary Material (ESM) Table 1).

All the DNA solutions were aliquoted and diluted to convenient concentrations using Milli-Q water unless otherwise stated. Double stranded DNA (dsDNA) templates were prepared by mixing equal volumes of complementary strands in phosphate buffered saline (PBS) buffer, heating to 95 ºC for 10 minutes and letting the solutions gently cool down to room temperature.

4.2. Experimental

4.4.1. Electrode preparation

The electrochemical platform consisted of a set of six square gold electrodes (1 mm2) on a 75 mm× 25 mm borosilicate glass slide substrate, as previously described [22]. The electrochemical detection set-up is depicted in Fig. S1 in the ESM.

4.4.2. Optimisation experiments

4.4.2.1. Vertical and lateral probe length spacing optimisation

The electrode arrays were functionalised by co-immobilisation of the forward primer having a poly-15T, a poly-30T, a poly-45T, a poly-30T/poly-30A or a poly-45T/poly45A of 2'-deoxythymidine monophosphate nucleotides at the 5' end of the probe as a vertical spacer with a range of probe-to-lateral spacer ratios: 1:1, 1:10 and 1:100. The dsDNA forward primers poly-30T/poly-30A and poly-45T/poly-45A were prepared as described in section 2. One microlitre of 10 µM thiolated forward primer of *F. tularensis* in 1 M KH_2PO_4 solution together with either 6-mercapto-1-hexanol, a thiolated poly-15T (with no primer) or a bipodal PEGylated thiol, 10-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9-trioxadecanol (DT1) [2] at 10 µM, 100 µM or 1000 µM, were drop-casted on the electrode surface. Control electrodes without lateral spacer for each of the primers with different length vertical spacers were also functionalised. Co-immobilisation was carried out in a saturated humidity chamber at room temperature for 20 h. Subsequently, the electrodes were rinsed in Milli-Q water and dried in a stream of nitrogen. A medical grade double-sided adhesive and a 3 mm thick PMMA block were aligned and pressure bonded with the functionalised electrodes to produce a fluidic chamber of 10 µl where amplification and detection was carried out. The PMMA gasket and the adhesive were cut and milled using a CO_2 laser marker (Fenix, Synrad, USA) to precisely define channel dimensions, as well as inlets and outlets in the PMMA cover plates. The chamber was then washed with 200 µl of PBS Tween 20, followed by 200 µl of Milli-Q water in order to completely remove any non-specific immobilisation of

DNA on the electrode surface. Subsequently, the chamber was incubated with Denhardt's solution at room temperature for 20 minutes in order to block the surface and prevent non-specific binding of SA-HRP or HRP-labelled reverse primer. The chamber was finally washed with 600 µl of PBS, 200 µl of Milli-Q, dried with a nitrogen beam and kept under vacuum until used (Solid-phase recombinase polymerase amplification

The RPA amplification mixture was prepared by mixing 2.4 µl of 10 µM biotinylated or horse radish peroxidase labelled reverse primer and 13.2 µl of 10 nM template dsDNA in 31.9 µl of rehydration buffer. Lyophilised pellets were then added and mixed by pipetting, before finally adding 2.5 µl of 280 mM magnesium acetate to trigger the reaction. The solution was then mixed by pipetting, 10 µl per array injected into each microfluidic channel of the set-up and left to incubate at 37 ºC for 1 h.

4.4.2.2. Amplification Temperature

Although the optimal temperature at which the strand-displacing polymerase *Bacillus subtilis* Polymerase I from the RPA enzymatic mixture is between 37 ºC and 38 ºC, the system has been reported to work in liquid at room temperature but at lower rates of amplification. To this end, the solid-phase RPA strategy has been tested between 35 ºC and 42 ºC, obtaining lower rates of amplification [13]. Solid-phase amplification (Section 3.2.2) was carried out at 22 ºC and 45 ºC and the electrochemical response measured to determine the amplification efficiency.

4.4.3. Electrochemical detection: chronoamperometry

Following completion of amplification, a series of different steps were carried out, each step of which was followed by flushing the microfluidic channels with 800 µL of PBS-Tween. In the case of the biotinylated reverse primer, 10 µL of 0.5 nM SA-HRP in PBS was added for 30 minutes, subsequent to amplification. The microfluidic channels were then filled with 10 µL of TMB enhanced one component HRP membrane substrate for 5 min prior to carrying out the electrochemical measurements. Finally, chronoamperometry was used to detect the oxidation of precipitated TMB by the HRP label, by applying a potential step from 0 V for 0.01 s to -0.2 V (the reduction potential of TMB) for 0.5 s to each electrode, sequentially, and taking the current readout at the end of the second step (*Fig. 4.1*). A potentiostat/galvanostat PGSTAT 12 Autolab controlled with the General Purpose Electrochemical System (GPES) was used to make the electrochemical measurements, and a home-made connection box used to plug the electrode arrays and generate electric connection between the potentiostat and the electrode arrays. In both of these strategies, a control measurement to ensure that all the signal readout was due to specific primer-to-target amplification was carried out, where the surface amplified duplex was denatured and then re-hybridised with biotin-labelled reverse primer followed by SA-HRP.

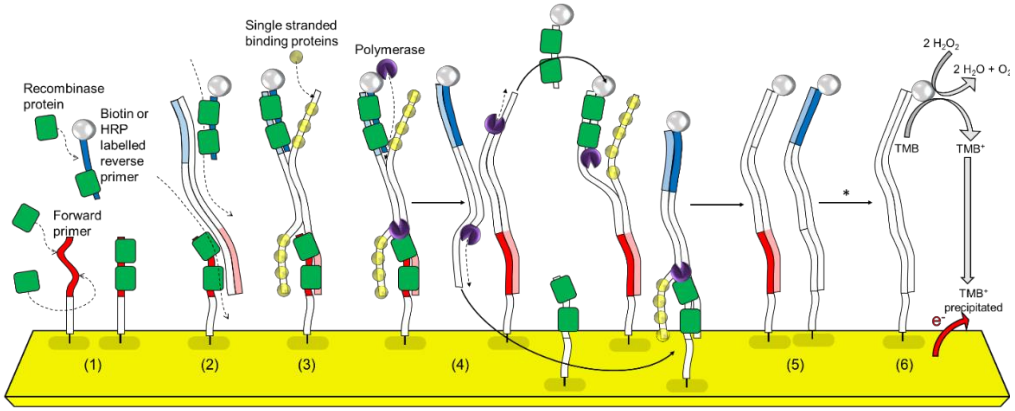

Figure 4.1 Solid-phase RPA with biotin or HRP labelled reverse primers. **(1)** Recombinase proteins form a complex with forward and reverse primers, **(2)** scan dsDNA for cognate sites and **(3)** introduce the primers in the template by a strand-displacement mechanism. **(4)** The polymerase initiates primer elongation at their 3' ends and exponential amplification is achieved by cycling of this process to produce **(5)** biotin or HRP labelled amplification products. **(*)** An additional conjugation step of SA-HRP is carried out in case a biotinylated primer was used during the RPA before electrochemical measurements. **(6)** Chronoamperometry is performed in the presence of tetramethylbenzidine (TMB) and H_2O_2 for the detection of precipitated and oxidised TMB substrate.

4.4.4. Calibration curve

The functionalisation of electrode arrays was carried out using the optimised protocol, *i.e.* drop-casting 1 µl per electrode of a KH_2PO_4 1 M solution containing the forward primer with a thiolated poly-15T and a primer-to-lateral spacer molar ratio of 6-mercapto-1-hexanol of 1:10 for 20 h in a water saturated chamber. The RPA amplification mixture was prepared as described in Section 3.2.2 with 5 different serial 1/10 dilutions of a *Francisella tularensis* dsDNA 10 nM template, including a blank control assay without template. A negative, *i.e.* non-specific

template, for the primers used here was already tested in a previous publication [14].

4.4.5. Real samples

DNA extracts from spleen and liver tissue from hares were kindly provided by the institute of Bacterial Infections and Zoonoses, Friendrich-Loeffler Institute. In brief, high Pure PCR Template Preparation Kit from Roche Diagnostics GmbH (Mannheim, Germany) was used for the DNA extraction. Pinhead-sized tissues samples were added to 200 µl tissues lysis buffer and 40 µl Proteinase K followed by incubation over night at 37 °C. 50 µl Proteinase K were added again and incubated at 50 °C for 1 h. 200 µl of the corresponding lysate were prepared according to the manufacturer's instructions for preparation of total nucleic acids from whole blood. The presence of *F. tularensis* DNA was amplified and determined by SP-RPA followed by electrochemical detection and the results were compared to polymerase chain reaction (PCR) followed by agarose gel electrophoresis. qPCR was also carried out to confirm the PCR and RPA results and to quantify the DNA contained in the real samples.

For the electrochemical detection, electrode arrays were modified according to the protocol described in section 3.4. The RPA amplification mixture was prepared as described in 3.2.2 replacing the 13.2 µL of 10 µM dsDNA by 2.5 µL of the DNA extract and 10.7 µL of PBS buffer.

PCR-Electrophoresis: PCR was performed according to indications provided by the Tfi DNA polymerase manufacturer. 50 µL of PCR cocktail was prepared including 200 µM dNTPs, 200nM of *F. tularensis* primers (without any modifications in 5'), 1.5 mM magnesium chloride, 5 units of *Tfi* DNA Polymerase and 2.5 µL of the sample. DNA

amplification process consisted of an initial denaturation step at 95 ºC for 5 min, followed by 25 cycles of 95 ºC for 30 s, 58 ºC for 30 s, and 72 ºC for 30 s and including a final elongation step at 72 ºC for 5 min. Amplification products were visualised by agarose gel electrophoresis. The gel was performed using ultra low pure agarose (3 %) in 1× Tris-Borate-EDTA buffer (TBE) and run for 35 minutes at 100 V.

qPCR: qPCR was performed according to indications provided in the PowerUp SYBR Green Master Mix. 10 µL of PCR cocktail was prepared including 5 µM PowerUp SYBR Green Master Mix, 10 µM primers, Nuclease-Free Water and 1 µL of the sample. DNA amplification consisted of an initial denaturation step at 95 ºC for 5 min, followed by 40 cycles of 95 ºC for 30 s, 58 ºC for 30 s, and 72 ºC for 30 s and including a final elongation step at 72 ºC for 5 min were performed on 7900HT Fast Real-Time PCR System. Calibration curves were constructed using 1 in 10 dilutions of 5nM synthetic target *F. tularensis* DNA.

4.5. Results and discussion

4.5.1. Vertical and lateral probe length spacing optimisation

Taking into account the heterogeneous format pursued, comprised of the solid-phase (the electrode), the liquid-phase (bulk solution) and the interface between these two phases (primers attached to the surface of the electrode), we defined different parameters affecting performance, focusing on the optimisation of the interface between bulk solution and the electrode, as this is where most of the amplification and detection occurs. First, the recombinase proteins form a complex with the primers and then scan the dsDNA target that is present in the

neighbourhood, this initial stage of amplification being critical for minimisation of amplification time. In the set-up used in the work reported here, a simple microfluidic channel defined the boundaries of the amplification chamber. Without applying any positive pressure, the typical flow gradient between the movement of particles in the bulk solution and the surface of the electrodes due to the friction of the liquid on the boundaries of the channel is of minor influence and the biological material present in the solution only experiences diffusion governed by specific hybridisation mechanisms such as base pairing and formation of complexes between proteins and oligomers. When considering the most important conditions to be optimised in the interface for improving amplification and detection, we hypothesised that the vertical and lateral probe length spacing were two parameters of importance. A longer vertical spacer between the electrode and the primer would be expected to enhance accessibility between the recombinases, polymerases, ssDNA binding proteins and the immobilised primers. Additionally, lateral spacers could be anticipated to reduce steric hindrance. However, vertically aligned probes (*i.e.* primers) are known to encourage target hybridisation as the negative charge of the backbone cause inter-primer repulsion, resulting in the probes being fully extended from the surface without folding or kinks. Thus, an optimal compromise between lateral primer spacing and primer-to-primer electrostatic repulsion is required. The combination of vertical and lateral spacers was thus evaluated, with the aim of achieving rapid D-loop formation, resulting in reduced amplification.

Table 4.1 shows a summary of results obtained after chronoamperometric measurements on arrays using the same

amplification conditions, evaluating different spacing configurations (vertical spacer, lateral spacing ratio and lateral spacer type), including controls with no lateral spacing for each vertical spacer used. First, it can be observed that the use of a poly-30T or poly-45T vertical spacer yielded unsatisfactory results when combined with no lateral spacer, and the same is true when a 1:1 primer-to-lateral spacer ratio was tested with each of poly-15T, DT1 or 6-mercapto-1-hexanol. This was anticipated and can be explained in terms of undesired interactions between neighbouring primers due to the extended length, a trend that is not observed when lateral spacing starts to be effective at higher ratios of 1:10 or 1:100. The shorter vertical spacer appeared to be short enough to avoid interaction between neighbouring primers, even when no lateral spacing was used. When using DT1 or 6-mercapto-1-hexanol at 1:100 primer-to-lateral spacer ratios the shortest vertical spacer yielded a much lower amplification than the longer vertical spacer counterparts, this effect is not observed at higher primer-to-lateral spacer ratios (1:1 and 1:10), so we suggest the lower amplification is due to the diminished surface coverage of the primer at 1:100 ratio. When using poly-15T as lateral spacer, the use of poly-30T and poly-45T as vertical spacers gave a poor but comparable yield to the poly-15T, and this can be attributed to be due to the specific nature of the lateral spacer employed. Poly-T is also a negatively charged strand and is expected to be in constant repulsion with the neighbouring strands, which could prevent longer vertical spacers from aggregating or interacting with neighbour primers. However, taking into account the reproducibility of the surfaces generated, the use of either 6-mercapto-1-hexanol or DT1, was preferable. Due to the fact that 6-mercapto-1-hexanol is a more

commonly used, more stable and more cost-effective option as compared to DTI, in further experiments it was used as lateral spacer at 1:10 ratio with immobilised forward primer. In summary, the enhancement in performance regarding the surface optimisation can be attributed to an improved access to the immobilised primers, which is achieved as a combination of the vertical spacer projecting the primer from the surface, thus facilitating elongation by the polymerase, whilst the lateral spacing avoids undesired interactions between neighbouring primers. Furthermore, vertical and lateral spacing of primers is expected to reduce steric hindrance between the proteins and the surface as well as between proteins during the amplification in solid phase.

Table 4.1 Chronoamperometric current density outputs (in $\mu A \cdot mm^{-2}$) (n = 8) for probe spacing optimisation using three different lateral spacers at different primer-to-lateral spacer molar ratios and three different primer vertical spacers.

Lateral spacer	Primer-to-lateral spacer molar ratio	Vertical spacer		
		15T	30T	45T
No Lateral Spacer	1:0	2.1 ± 0.7	0.7 ± 0.2	0.8 ± 0.6
Poly-15T	1:1	2.5 ± 1.1	1.7 ± 0.3	1.5 ± 0.9
	1:10	2.6 ± 1.0	1.5 ± 0.3	1.7 ± 0.8
	1:100	1.7 ± 1.0	1.7 ± 0.3	0.8 ± 0.9
6-mercapto-1-hexanol	1:1	3.4 ± 0.2	0.70 ± 0.14	0.6 ± 0.2
	1:10	4.1 ± 0.2	3.3 ± 0.2	3.7 ± 0.3
	1:100	1.0 ± 0.2	3.0 ± 0.2	3.3 ± 0.3
DT1	1:1	3.2 ± 0.3	0.8 ± 0.2	0.9 ± 0.2
	1:10	3.5 ± 0.3	3.9 ± 0.2	4.3 ± 0.2
	1:100	1.6 ± 0.3	4.3 ± 0.2	4.7 ± 0.2

The effect of the vertical spacer stiffness on the forward primers was also assessed by using the ssDNA vertical spacers: poly-30T and poly-45T, and the dsDNA vertical spacers: poly-30T/30A and poly-45T/45A. Poly-15T/15A was not studied as the melting temperature of this duplex is below that of the amplification temperature of at least 37 ^{o}C used for amplification. *Table 4.2* compares the current density obtained with the dsDNA vertical spacer respect the current obtained with the ssDNA vertical spacer, expressed in %. Results show in most cases a decrease in the current output when using the dsDNA vertical spacer compared to the current output using ssDNA as vertical spacer. This could be explained by the effect of two different factors; first, the dsDNA being twice as thick as the ssDNA and therefore spacing out the primers more on the surface of the electrode, thus reducing the amount of receptors as well as the current output. Second, a poly-A tail does not necessarily perform a complete hybridisation with all the nucleotides with a complementary poly-T sequence. This could lead to a partial hybridisation between the poly-T and the poly-A sequences, allowing the formation of poly-A tails at the 5’ or 3’ ends of the poly-T spacers. While the poly-A tail at the 5’ end could interfere with the formation of the thiol-Au bond, the poly-A tail at the 3’ end could impede the access to the polymerase, both decreasing the final amplification and inherently the current output.

Table 4.2 Comparative study showing % of current density output using dsDNA vertical spacers versus ssDNA vertical spacers.

Lateral spacer	Primer-to-lateral spacer molar ratio	% of the current density output	
		30T/30A *vs.* 30T	**45T/45A *vs.* 45T**
6-mercapto-1-hexanol	1:1	100	87
	1:10	110	88
	1:100	100	100
DT1	1:1	79	69
	1:10	80	91
	1:100	89	100

4.5.2. Optimisation of amplification temperature

The SP-RPA strategy was tested at 45 ºC and 22 ºC (room temperature) in addition to the optimal working temperature for the polymerase, *i.e.* 37 ºC, for comparative purposes. The output current density recorded of the process after 1 h of amplification was -3.5 ± 0.3 μA·cm^{-2} (n = 4) at 37 ºC, while the measurements recorded at 45 ºC and 22 ºC did not yield a significant output current, 0.7 ± 0.2 μA·cm^{-2} (n = 4) and 0.6 ± 0.3 μA·cm^{-2} (n = 4) respectively, and is comparable to the output current densities of control electrodes, indicating that the amplification efficiency was close to 0 %, although some degree of amplification was expected at least at 22 ºC. *Bacillus subtilis* Polymerase I has an optimal activity at 37 ºC and besides a reduced activity of the polymerase at non-optimal temperatures, solid-phase RPA strategy adds a singular constraint to the system by anchoring the forward primers on a solid substrate, not only limiting the mobility of the primers, but also requiring the proteins involved to move from the bulk solution to the surface. This effect has been studied by [13] where a 5

ºC shift from the ideal temperature already yielded a loss in performance of 60 % and 40 % for liquid and solid-phase amplification, respectively.

4.5.3. Labelled reverse primer performance and calibration curve

Solid-phase amplification and detection using either a biotin-labelled reverse primer or a HRP-labelled reverse primer resulted in similar performances. In both cases the chronoamperometric response, in the presence of a TMB/H_2O_2 solution for 5 minutes, was also recorded after denaturation of the surface of the electrodes, showing no signal output, and recorded again after hybridisation with a biotin-labelled reverse primer and complex formation with SA-HRP, showing comparable results to the first responses obtained (*Fig. 4.2*). This confirms that the signal obtained is due to solid-phase amplification, and additionally that the signal due to non-specific binding of the HRP-labelled primer on the surface is negligible.

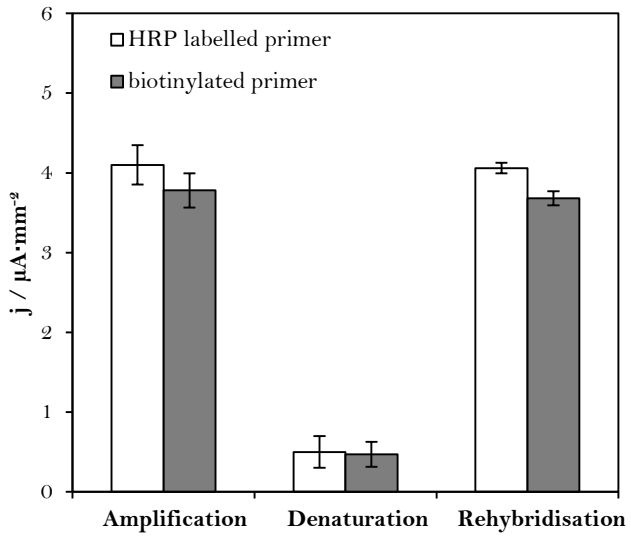

Figure 4.2 Chronoamperometric current recorded in TMB/H_2O_2 substrate after the RPA using biotin or HRP-labelled reverse, after denaturation with NaOH 0.1 M and rehybridisation with a biotin-labelled reverse primer/conjugation with streptavidin-HRP again.

The results obtained are summarised in *Table 4.3* and compared with our previously reported work, where we used a different surface chemistry and a labelling strategy involving regular primers during solid-phase RPA followed by denaturation, hybridisation with biotinylated primers and addition of SA-HRP. Using HRP-labelled primer or biotinylated reverse primers facilitated a huge improvement in terms of protocol simplicity (avoiding up to 3 or 4 steps) and analysis time required (reduced by 50 %), with more reproducible results obtained due to the fact that many manual steps are omitted, whilst the performance obtained using a non-optimised surface chemistry (no lateral spacer used and RPA performed at 37ºC) for both biotin and HRP-labelled primers yielded a lower sensitivity (~50 % less), lower reproducibility and LODs two orders of magnitude higher than the results obtained for the optimised surface strategies (*Figure 4.3*, Raw data Supplementary

Information Figure SI2). The LOD was also higher than even previously reported [14], and can be attributed to non-specific binding of the SA-HRP protein onto the surface, as no alkanethiol was used in the mixed self-assembled monolayer (SAM).

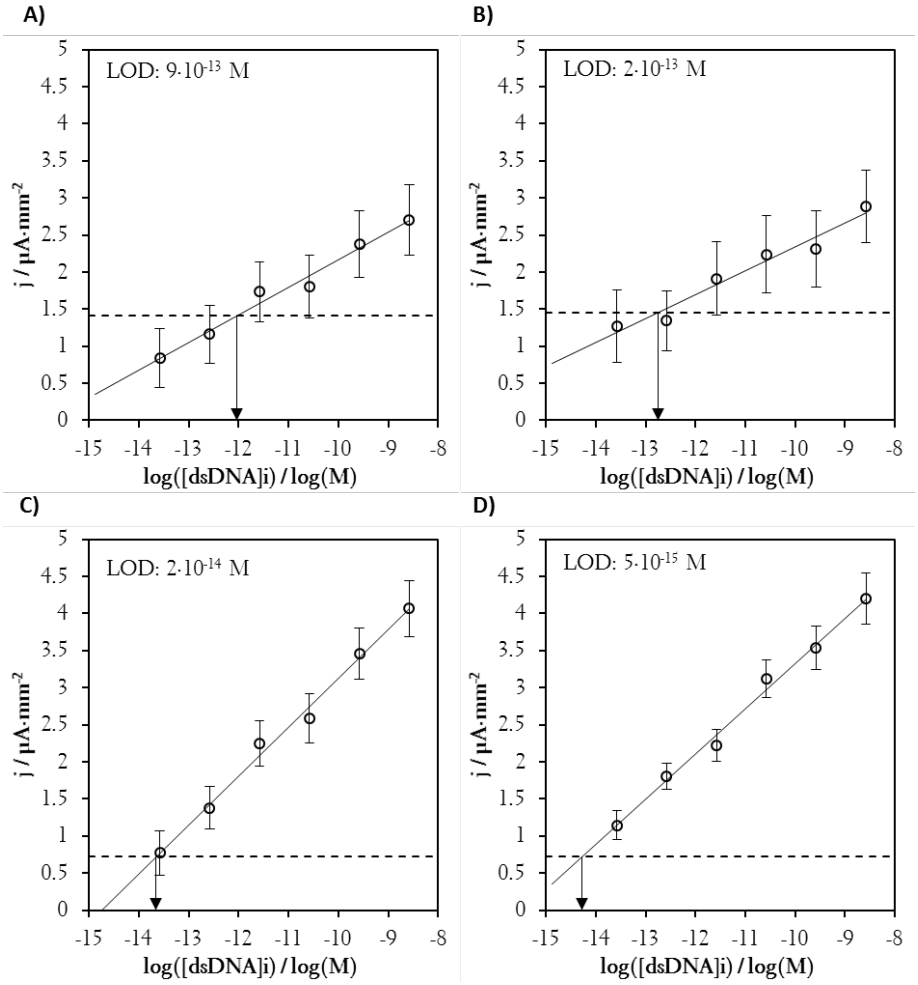

Figure 4.3 Calibration curves of the **A)** biotinylated primer with non-optimised surface **B)** HRP-reverse primer with non-optimised surface **C)** biotinylated reverse primer with optimised surface and **D)** HRP-labelled reverse primer with optimised surface

Table 4.3 Analytical parameters.

	Previous work†	Non-optimised surface		Optimised surface	
		Biotin-primer	**HRP-primer**	**Biotin-primer**	**HRP-primer**
Sensitivity ($\mu A \cdot mm^{-2} \cdot M^{-1}$)	0.14	0.37	0.32	0.65	0.61
LOD (M)*	$3.3 \cdot 10^{-14}$	$9.1 \cdot 10^{-13}$	$1.7 \cdot 10^{-13}$	$2.2 \cdot 10^{-14}$	$5.3 \cdot 10^{-15}$
Linear range (log(M))	-8.6 to -13.6	-9.6 to -12.6	-9.6 to -12.6	-8.6 to -12.6	-8.6 to -12.6
Coefficient of variation (%)	22.5	27.4	26.1	17.5	10.2
Analysis time (h)	3.0	2.0	1.5	2.0	1.5

†Solid-phase recombinase polymerase amplification [14]
* LOD calculated by the 3 times standard deviation of the blank method

4.5.4. Real samples

The presence of *F. tularensis* was determined in real samples by SP-RPA using biotinylated primers and electrochemical detection and the results were compared with PCR and agarose gel electrophoresis. Sample 1 and 2 (negative samples) were extracted from spleen tissue and samples 3 and 4 (positive samples) from liver tissue. *Figure 4.4* shows the results obtained by both methods. The results obtained by SP-RPA were analysed using Student's t test. Only samples 3 and 4 showed statistical difference compared to control for a significance level of 0.05, thus representing positive results and corroborating the results obtained by PCR-gel electrophoresis, as well as qPCR analysis (Supplementary Information, Figure SI3, SI4, SI5). qPCR was used to calculate the concentration of *F.tularensis* DNA in the extracts and found to be 500fM, highlighting the ability of RPA to detect these low levels of DNA in real samples.

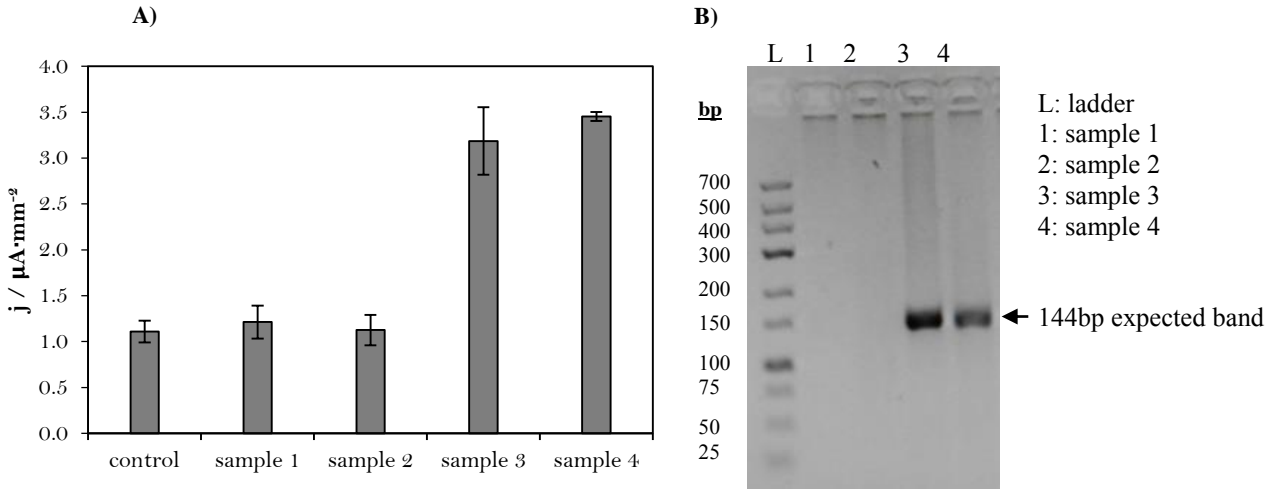

Figure 4.4 A) Chronoamperometric current recorded in TMB/H_2O_2 substrate after the RPA using biotin reverse primer, for a control with no target and 4 different samples (n=3). **B)** Agarose gel obtained after PCR amplification of the same samples.

4.6. Conclusions

We previously reported the exploitation of solid-phase recombinase polymerase amplification as a DNA detection system. Here, we extended this work pursuing alternative strategies in the construction of an electrochemical platform for the detection of DNA, simplifying the protocol and reducing the assay time required. The amplification-detection platform was optimised by addressing various aspects, including fine-tuning of the surface chemistry via optimisation of the vertical and lateral spacing of the immobilised primers in order to obtain lower LODs and maximal signal-to-noise ratio. The use of biotin and HRP labelled primers were also evaluated, thus avoiding two or three steps, respectively and inherently significantly simplifying the assay. Furthermore, the improvement of the electrode design facilitated a higher signal-to-noise ratio, which, in combination with the optimised

protocol resulted in a vastly improved amplification-detection protocol, reducing the assay by 1.5 h and obtaining a LOD in the range of $1 \cdot 10^{-15}$ M. Finally, we demonstrated the viability of the setup to determine the presence of *F. tularensis* in real samples, which was validated using PCR and qPCR, demonstrating the ability of the developed solid-phase RPA to detect 500fM DNA in real samples.

4.7. Acknowledgements

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4.8. Compliance with Ethical Standards

The authors declare no conflict of interest.

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CHAPTER 5

Synthesis of Aminophenyl-, Nitrophenyl- and Benzofurazane - labelled nucleoside triphosphates

UNIVERSITAT ROVIRA I VIRGILI
DEVELOPMENT OF DIAGNOSTIC PLATFORM FOR DETECTION OF BIOLOGICAL AGENTS AND TOXIC MICROALGAE USING ISOTHERMAL AMPLIFICATION.
Olena Mayboroda

Synthesis of Aminophenyl-, Nitrophenyl- and Benzofurazane - labelled nucleoside triphosphates

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Keywords nucleoside triphosphates; synthesis; cross-coupling; triphosphorylation.

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5.1. Abstract

Redox-labelled nucleotides are garnering increasing interest for the development of next generation molecular tools and should meet requirements of being thermally stable, sensitive, and compatible with polymerase-mediated incorporation whilst also being electrochemically active. Here we present the synthesis strategies for aminophenyl-, nitrophenyl- and benzofurazane-modified dNTPs for their exploitation via enzymatic incorporation into elongating DNA, and the subsequent electrochemical detection of specific DNA sequences.

5.2. Introduction

Naturally nucleic acids are electroactive by themselves [1,2], but this detection approach does not provide enough specificity to differentiate one target from another one. A further possible detection method can use various redox modified dN^{X}TPs that get incorporated during the elongation process as building blocks of DNA. Newly amplified DNA containing functionalised nucleobases have immense potential for applications in chemical biology, nanotechnology, material science, as well as bioanalysis. Since the 1990s, there have been multiple reports on the synthesis of labelled dN^{X}TPs including amino- and thiol- [3], imidazoyl-moieties [4] for the preparation of modified DNA. More recently, other redox-labels have been reported, including amino- and nitrophenyl- [5], ferrocene [6], Ru- or Os(bpy) complexes [7], alkylsulfanylphenyl [8], anthraquinone [9], benzofurazane [10], azidophenyl [11], and polyoxometalates [12].

Modified dN^{X}TPs have found several applications in modern chemical biology, each of them offering their own advantages and

limitations. For example, ferrocenes are prone to oxidation by air, the $dN^{X}TPs$ bearing bulky inorganic $[Os(bpy)_3]^{2+}$ are poor substrates for DNA polymerases, and the redox potential of Os^{2+} is close to that of 7-deazaguanine base [9,13]. Nevertheless, the choice of redox labels is still wide enough, so the proper label can be selected for each application and system.

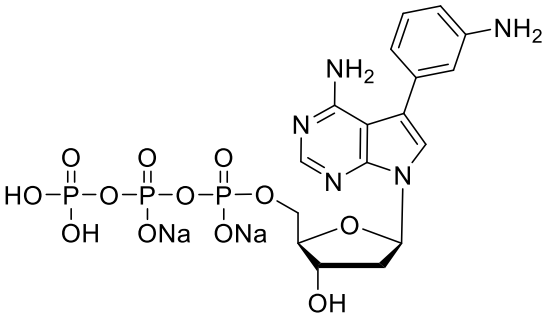
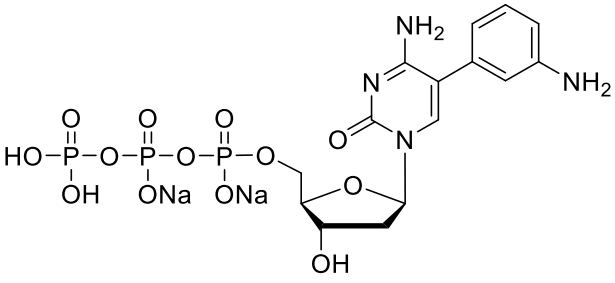
Labelled $dN^{X}TPs$ can be prepared using two main methodologies. In the classic approach, functionalised $dN^{X}TPs$ are obtained by primer cross-coupling of a halogenated 2'-deoxynucleoside (dN^{I}) followed by triphosphorylation of the resulting modified nucleoside to obtain modified $dN^{X}TPs$. In 1967, Yoshikawa *et al.* [14] described the first method for the synthesis of $dN^{X}TPs$ using triphosphorylation. Briefly, the procedure involved selective 5'-mono-phosphorylation of an unprotected nucleoside with phosphorous oxychloride yielding 5'-phosphorodichloridate intermediate that is subsequently treated *in situ* with tributylammonium pyrophosphate to yield a cyclic triphosphate, which is subsequently hydrolysed to the desired $dN^{X}TPs$. Another protocol for triphosphorylation was described by Ludwig and Eckstein [15], where 5'-O-triphosphorylation of 2'-deoxynucleosides was achieved using salicyl chlorophosphite in the first step. The cyclic intermediate is then further oxidised by iodine and hydrolysed to the desired $dN^{X}TP$. This approach has higher specificity as compared to the Yoshikawa method.

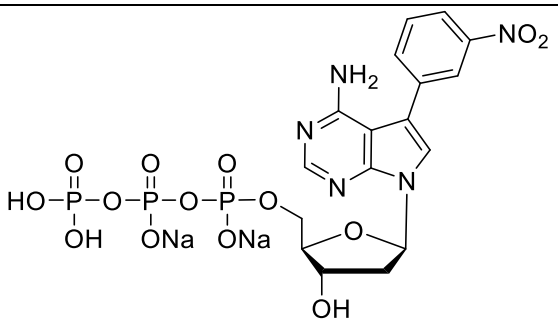
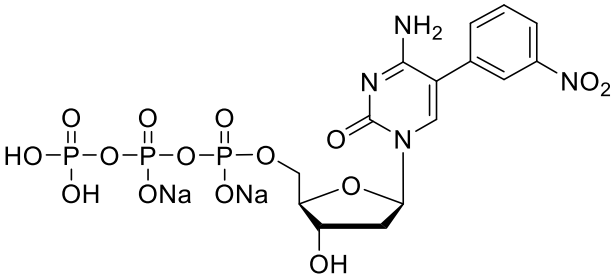
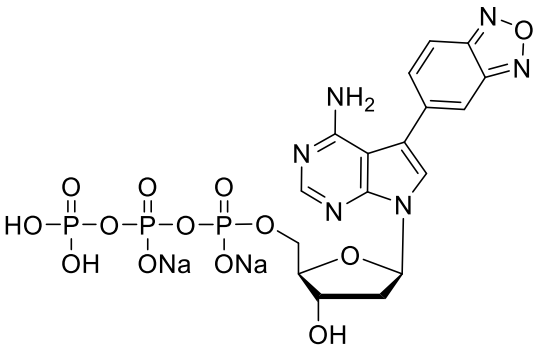
The second and more straightforward approach is a direct aqueous cross-coupling reaction of halogenated $dN^{I}TPs$. Cross-coupling reactions are in general very tolerant to most reactive functional groups. This methodology was facilitated by the development of water soluble phosphine ligands [16]. Several coupling methods have been reported

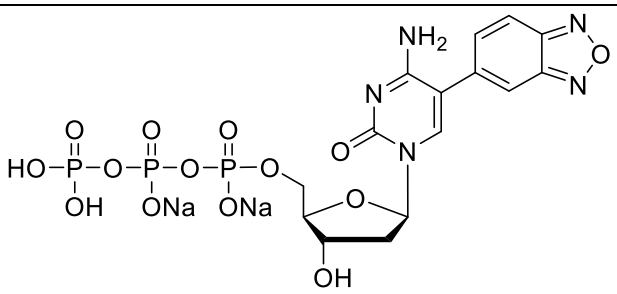
including Sonogashira coupling [17], Suzuki–Miyaura coupling [18] and the Heck reaction [19]. The main drawback of direct dN^{I}TPs modification using cross-coupling is the hydrolysis of triphosphates with increasing temperature. Therefore, the reaction should be optimised to be completed within 30–60 min.

In this work, we exploited two strategies for the synthesis of amynophenyl-, nitrophenyl- and benzofurazane-labelled dN^{X}TPs for subsequent incorporation in DNA by primer extension (PEX) (*Figure Table 5.1*) [5,10].

Table 5.1 Chemical structure of labelled nucleoside triphosphates (dNTPs).

dA^{NH2}TP	 Chemical Formula: $C_{17}H_{20}N_5Na_2O_{12}P_3$ Molecular Weight: 625.27
dC^{NH2}TP	 Chemical Formula: $C_{15}H_{19}N_4Na_2O_{13}P_3$ Molecular Weight: 602.23

dA^{NO2}TP	 Chemical Formula: $C_{17}H_{18}N_5Na_2O_{14}P_3$ Molecular Weight: 655.25
dC^{NO2}TP	 Chemical Formula: $C_{15}H_{17}N_4Na_2O_{15}P_3$ Molecular Weight: 632.21
dA^{BF}TP	 Chemical Formula: $C_{17}H_{17}N_6Na_2O_{13}P_3$ Molecular Weight: 652.25

dC^{BF}TP	 Chemical Formula: $C_{15}H_{16}N_5Na_2O_{14}P_3$ Molecular Weight: 629.21

5.3. Synthesis of modified nucleotides

5.3.1. Suzuki–Miyaura cross-coupling of the corresponding nucleosides followed by triphosphorylation

A 2:1 water-acetonitrile mixture (1 mL) was added to an argon-purged flask containing halogenated nucleoside dN^I (1 equiv), boronic acid (3 equiv), and Cs_2CO_3 (3 equiv). In a separate flask, $Pd(OAc)_2$ (0.05 equiv) and TPPTS (2.5 equiv with respect to Pd) were mixed, the flask was vacuumed and purged with argon, and then a 2:1 mixture of H_2O/CH_3CN (0.5 mL) was added. The catalyst solution was then injected into the reaction mixture, which was stirred at 60 °C for 1–2 h until the complete consumption of the starting material. The products dN^X were purified by column chromatography on silica gel eluting with chloroform/methanol (0 to 10% v/v).

Modified previously nucleoside dN^X (1 equiv) was placed into a flask and dried under vacuum at 60 °C for 1 h. The dry compound was suspended in trimethyl phosphate (0.5 ml) at 0°C and $POCl_3$ (1.5 equiv) was added. After 1.5 h, an ice-cooled solution of $(NHBu_3)_2H_2P_2O_7$ (5 equiv) and Bu_3N (4 equiv) in dry DMF (1 ml) was added and the mixture

was stirred at 0°C for another 2 h. The reaction was quenched with 2M TEAB buffer (2 mL). The solvents were evaporated in vacuum and the residue was co-distilled with water three times. The product was purified by HPLC on a C18 column, eluting with a linear gradient from 0.1M triethylammonium bicarbonate (TEAB) in H_2O to 0.1M TEAB in H_2O/MeOH (1:1). The final product was converged to the sodium salt form (Dowex 50WX8 in Na^+ cycle) and freeze-dried in order to get a solid product dN^XTP.

5.3.2. Suzuki–Miyaura cross-coupling of dN^ITPs

A 2:1 H_2O/CH_3CN mixture (1 mL) was added to an argon-purged flask containing halogenated nucleotide dN^ITP (1 equiv), boronic acid (3 equiv), and Cs_2CO_3 (3 equiv). In a separate flask, a mixture of $Pd(OAc)_2$ (0.05 equiv) and TPPTS (2.5 equiv with respect to Pd) was vacuumed and purged with argon, and then a 2:1 mixture of H_2O/CH_3CN (0.5 mL) was added. Subsequently, the catalyst solution was injected into the reaction mixture, which was then stirred at 60 °C for 1 h until the complete consumption of the starting material. The final dN^XTPs were purified by HPLC on a C18 column, eluting with a linear gradient from 0.1M triethylammonium bicarbonate (TEAB) in H_2O to 0.1M TEAB in H_2O/MeOH (1:1). The final product was converged to the sodium salt form (Dowex 50WX8 in Na^+ cycle) and freeze-dried in order to get a solid product.

5.3.3. PEX protocol

Primer (3 μM) was mixed with template DNA (3 μM), dNTPs (200 μM each) and KOD XL polymerase. The reaction mixtures were incubated

for 40 minutes at 60 °C and 2 minutes at 95 °C. The PEX products were mixed with loading buffer (80% formamide, 10 mm ethylenediaminetetraacetic acid (EDTA), 1 $mgmL^{-1}$ xylene cyanol, 1 $mgmL^{-1}$ bromophenol blue) and subjected to electrophoresis in 12% w/v denaturing polyacrylamide gel containing 1xTBE buffer (pH 8) and 7M urea at 25W for 50 min. Gels were dried, autoradiographed and visualised using Phosphorimager Storm.

5.4. Results and discussion

The modified $dN^{X}TPs$ were prepared using two different approaches: A. modification of the corresponding nucleoside followed by triphosphorylation and B. direct cross-coupling reaction (*Figure 5.1*) in analogy to previously developed procedures [20,21].

Directly linked NH_2 derivatives of nucleosides dA^{NH2} and dC^{NH2} were prepared, achieving good yields of 71 – 89 % in one step by Suzuki–Miyaura cross-coupling using dA^{I} or dC^{I} as initial substrates. Suzuki–Miyaura cross-coupling of halogenated $dN^{I}TPs$ ($dA^{I}TP$ and $dC^{I}TP$) under the same aqueous conditions did not give the desired NH_2-modified dNTPs ($dA^{NH2}TP$ and $dC^{NH2}TP$), so we applied an alternative strategy of triphosphorylation [22] of the corresponding nucleosides (dN^{NH2}) achieving a yield of 25%.

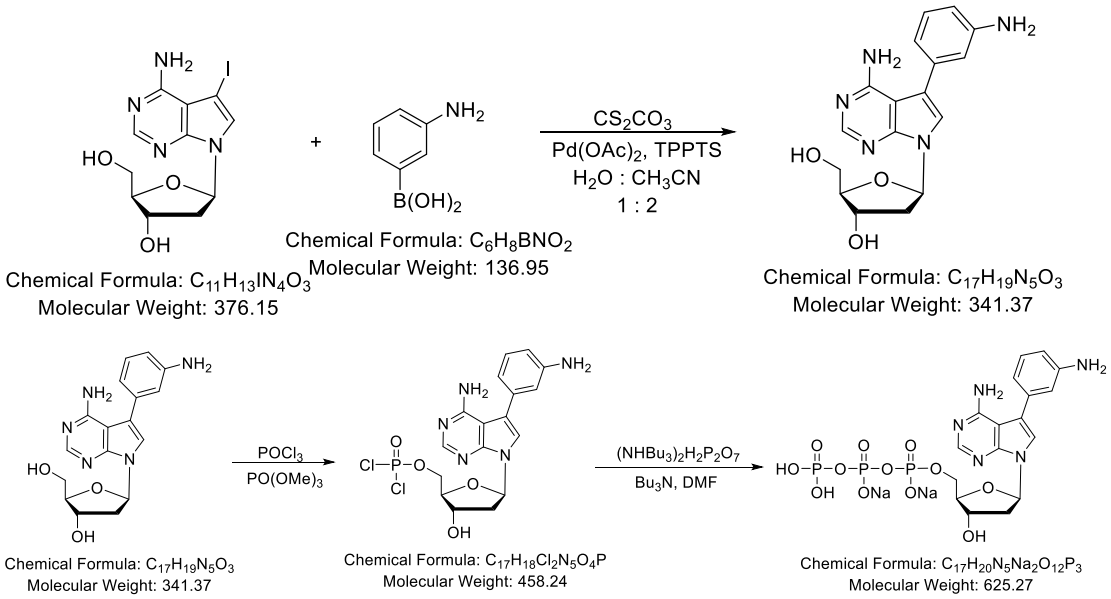
Directly linked NO_2 derivatives of nucleosides dA^{NO2} and dC^{NO2} were prepared, achieving good yields of 71.5 – 91 % in one step by Suzuki– Miyaura cross-coupling using dA^{I} or dC^{I} as initial substrates. Suzuki–Miyaura cross-coupling of halogenated $dN^{I}TPs$ ($dA^{I}TP$ and $dC^{I}TP$) under the same aqueous conditions did not give the desired NO_2-modified dNTPs ($dA^{NO2}TP$ and $dC^{NO2}TP$), so we applied an alternative strategy of

triphosphorylation of the corresponding nucleosides (dN^{NO2}) achieving a yield of 30%.

Directly linked BF derivatives of nucleosides dA^{BF} and dC^{BF} were prepared, with good yields of 70 – 90 % in one step by Suzuki– Miyaura cross-coupling using dA^{I} or dC^{I} as initial substrates. Suzuki–Miyaura cross-coupling of halogenated dN^{I}TPs (dA^{I}TP and dC^{I}TP) under the same aqueous conditions gave the desired BF-modified dNTPs (dA^{BF}TP and dC^{BF}TP), with a yield of 71%. We then applied an alternative strategy for triphosphorylation of the corresponding nucleosides (dN^{BF}) achieving a comparable yield of 75 %.

These straightforward and efficient approaches gave the desired functionalised dN^{X}TPs, which are suitable as substrates for polymerase incorporation.

A)



B)

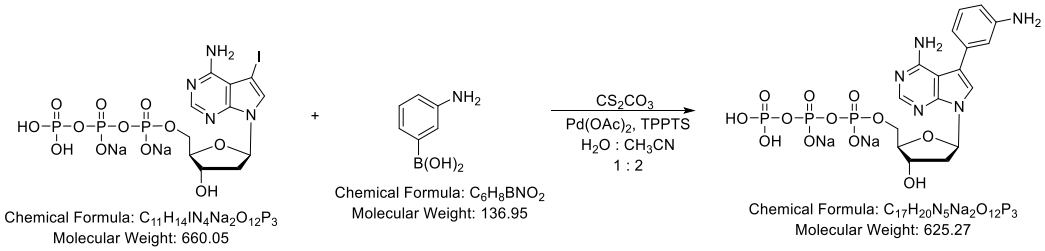


Figure 5.1 General strategy for the synthesis of modified dA^{NH2}TP by (**A**) modification of the corresponding nucleoside followed by triphosphorylation and (**B**) direct cross-coupling reaction

All conjugates were successfully incorporated into oligonucleotides through a primer extension (PEX) reaction catalysed by KOD XL DNA polymerase (*Figure 5.2*).

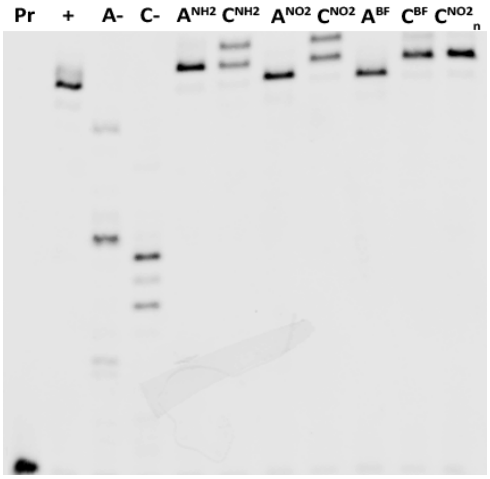


Figure 5.2 PEX incorporation of labelled dNTPs into one ON by KOD XL. **P**: primer; **+**: natural dNTPs; **A^{-}**: dCTP, dGTP, dTTP; **C^{-}**: dATP, dGTP, dTTP; **A^{NH2}**: dA^{NH2}TP, dCTP, dGTP, dTTP; **C^{NH2}**: dC^{NH2}TP, dATP, dGTP, dTTP; **A^{NO2}**: dA^{NO2}TP, dCTP, dGTP, dTTP; **C^{NO2}**: dC^{NO2}TP, dATP, dGTP, dTTP; **A^{BF}**: dA^{BF}TP, dCTP, dGTP, dTTP; **C^{BF}**: dC^{BF}TP, dATP, dGTP, dTTP; **C$^{NO2}_n$**: new batch of dC^{NO2}TP, dATP, dGTP, dTTP.

5.5. Conclusions

In conclusion, we have applied two of the most common synthesis strategies to achieve aminophenyl-, nitrophenyl- and benzofurazane-containing dN^{X}TPs. In all cases, the best performing method was the Suzuki–Miyaura cross-coupling of the corresponding nucleosides followed by triphosphorylation, resulting in the desired products with relatively good yields. The modified dN^{X}TPs were further efficiently incorporated by KOD XL DNA polymerase to form NH_2, NO_2 or BF-modified oligonucleotides. This gives us the possibility to further use these dN^{X}TPs in DNA amplification and electrochemical detection.

5.6. Acknowledgements

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CHAPTER 5

CHAPTER 6

Thermal and isothermal DNA amplification and detection system using Aminophenyl-labelled nucleoside triphosphates

UNIVERSITAT ROVIRA I VIRGILI
DEVELOPMENT OF DIAGNOSTIC PLATFORM FOR DETECTION OF BIOLOGICAL AGENTS AND TOXIC MICROALGAE USING ISOTHERMAL AMPLIFICATION.
Olena Mayboroda

CHAPTER 6

Thermal and isothermal DNA amplification and detection system using Aminophenyl-labelled nucleoside triphosphates

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Keywords thermal and isothermal DNA detection, electrochemistry, labelled nucleoside triphosphates, toxic microalgae

UNIVERSITAT ROVIRA I VIRGILI
DEVELOPMENT OF DIAGNOSTIC PLATFORM FOR DETECTION OF BIOLOGICAL AGENTS AND TOXIC MICROALGAE USING ISOTHERMAL AMPLIFICATION.
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6.1. Abstract

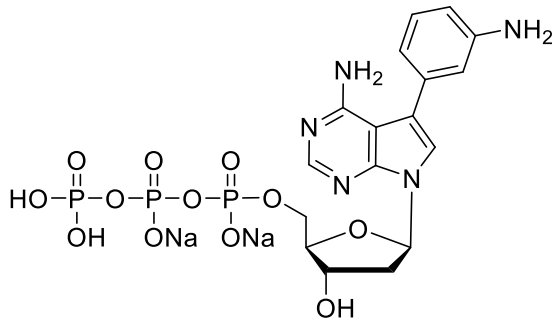
Redox-labelled nucleotides have been garnering increasing interest for the development of next generation molecular tools and should meet requirements of being thermally stable, sensitive, and compatible with polymerase-mediated incorporation whilst also being electrochemically active. Aminophenyl-modified dN^{X}TPs were synthesised and used for thermal and isothermal polymerase based amplification of a DNA sequence specific for *Karlodinium armiger.* This work demonstrates for the first time, 100 % incorporation of labelled dN^{X}TPs using isothermal recombinase polymerase amplification. Specifically designed primers were used to generate double-tail single stranded DNA amplicons suitable for direct detection via hybridisation using either optical or electrochemical transduction.

6.2. Introduction

DNA biosensors and chips are widely researched for use in molecular diagnostics. Electrochemical detection is a less expensive but comparatively sensitive alternative to common optical methods. Even though nucleic acids are electroactive themselves [1,2], diverse electroactive tags are used to increase sensitivity and specificity. DNA containing functionalised nucleobases have immense potential for applications in chemical biology, nanotechnology, material science, as well as bioanalysis. Since 1990s, there were multiple reports on the synthesis of labelled dN^{X}TPs including amino and thiol [3], imidazoyl moieties [4] for the preparation of functionalised DNA. More recently, other redox-labelled dN^{X}TPs have been reported, including amino- and nitrobenzene [5], ferrocene [6], Ru- or Os(bpy) complexes [7],

alkylsulfanylphenyl [8], anthraquinone [9], benzofurazane [10], azidophenyl [11] and polyoxometalate [12].

The thorough choice of the appropriate redox label has to be done depending on the detection system due to several limitations of some modified dN^{X}TPs: ferrocenes are prone to oxidation by air, the dN^{X}TPs bearing bulky inorganic [Os(bpy)3] are poor substrates for DNA polymerases, and the redox potential of Os^{2+} is close to that of 7-deazaguanine base [9,13]. Nevertheless, the choice of redox labels is still wide enough, so the proper label can be selected for each application and system. After evaluating several redox labels like aminophenyl-, nitrophenyl- and benzofurazane-labelled dN^{X}TPs, aminophenyl-labelled dATP was chosen (*Figure 6.1*) [5] for this work. It has a high level of incorporation, the size of the modification is relatively small and the measurement potential range of this modified dN^{X}TP is appropriate as an electrochemical label for biosensor applications (a low potential is required, thus not affecting the stability of the chemical platform and the biomolecules immobilised on the surface of the electrode).



Chemical Formula: $C_{17}H_{20}N_5Na_2O_{12}P_3$
Molecular Weight: 625.27

Figure 6.1 Chemical structure of Aminophenyl-labelled adenosine triphosphate (dA^{NH2}TP).

There are several methods of polymerase based synthesis of DNA. One of the most basic ways to produce short double stranded DNA (dsDNA) modified in one strand is primer extension (PEX). This is an isothermal reaction suitable for particular DNA polymerases such as the Klenow fragment of *Escherichia coli*. PCR is used for preparation of longer DNA with a higher level of modifications in both strands. The temperatures and times of individual steps depends on the sequence, the length, the sequence of the primers, the type of DNA polymerase used, and and the types modifications of $dN^{X}TPs$. Thus, every modification requires an individualised optimisation of conditions.

Whilst PCR has revolutionised molecular diagnostics, facilitating the rapid amplification of DNA, there are several limitations of PCR for implementation in a point of care (POC) or point of need (PON) device [14,15], including the need for thermal cycling, risk of contamination, the relatively high cost of equipment and a need for trained personnel. To overcome these limitations, isothermal amplification techniques have been developed, such as loop mediated isothermal amplification (LAMP) [16], nucleic acid sequence based amplification (NASBA) [17], helicase-dependent amplification (HDA) [18], strand displacement amplification (SDA) [19], rolling circle amplification (RCA) [20] and recombinase polymerase amplification (RPA) [21,22]. Of all the isothermal techniques developed to date, recombinase polymerase amplification, RPA,[23] deserves special attention. It operates at a stable isothermal temperature (37–42 °C), without a need for an initial DNA melting step, and amplification can be achieved in just 5-10 min.

The combination of isothermal RPA with redox labelled $dN^{X}TPs$ for the amplification and detection of toxic microalgae is an innovative

method that permits incorporation of labels throughout the amplification process facilitating direct electrochemical detection of the RPA products and an inherent shortening of assay time. The presence of redox labels through the whole length of amplicon will also facilitate significant signal enhancement, allowing lower limits of detection.

To simplify the detection of the amplicon, a tailed primer approach developed by Joda *et al.* [24] was used. This primer design results in an amplicon product flanked by two single stranded DNA tails (*Figure 6.2*). These tails are used for direct hybridisation with a surface immobilised probe. The use of modified primers for the production of dsDNA that can be directly detected is cost effective, efficient, robust and easily multiplexed. Moreover, this approach brings us closer to an ASSURED "sample-in response-out" system that should be as simple as possible with a minimum number of steps, reagents and end-user intervention.

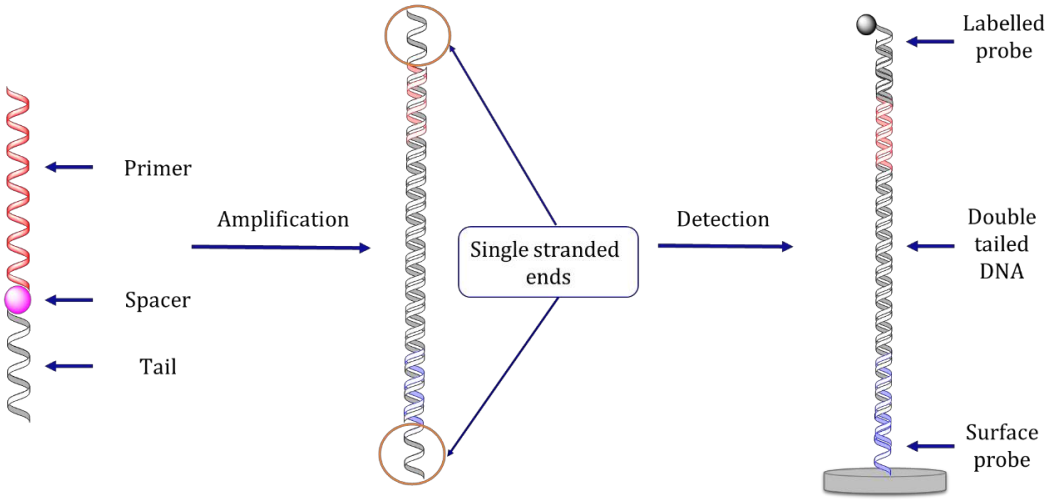


Figure 6.2 Schematic of amplicon detection using the tailed primers design.

6.3. Materials and methods

6.3.1. Chemicals

RPA kit TwistAmp® Basic was obtained from TwistDx Ltd. (Babraham, United Kingdom). The HRP substrate formulation tetramethylbenzidine (TMB) enhanced one component HRP membrane was purchased from Diarect AG (Germany), GelRedTM Nucleic Acid Gel Stain from Biotium (Barcelona, Spain), certified molecular biology agarose gel powder from Bio Rad Laboratories S.A. (Barcelona, Spain), Klenow (exo-) polymerase from ThermoFisher (Barcelona, Spain) and KOD XL DNA polymerase from Merck Chemicals & Life Science S.A. (Madrid, Spain). Maleimide-activated microtitre plates were obtained from ThermoScientific (Madrid, Spain). Streptavidin–peroxidase from *Streptomyces avidinii* and peroxidase substrate 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate system for ELISA were purchased from Sigma-Aldrich S.A. (Barcelona, Spain). Labelled $dN^{X}TPs$ were kindly synthesised in the Bioorganic and Medical Chemistry of the Nucleic Acids Group in the Institute of Organic Chemistry and Biochemistry Academy of Sciences of the Czech Republic. All other chemicals were obtained from Sigma Aldrich S.A. (Barcelona, Spain) and used as received.

6.3.2. DNA sequences

Synthetic oligonucleotides were obtained as lyophilized powder from Biomers.net (Ulm, Germany).

K. armiger tailed forward primers (FwP):

5'- ATT ACG ACG AAC TCA ATG AA - * - ATA GCT TCA CAG CAG AGG TTA CAA C -3'

K. armiger tailed reverse primers (RvP):

5'- TGT AAA ACG ACG GCC AGT - * - ACA CAC ATC CAA CCA TYT CAC TG - 3'

Template *K. armiger* DNA (153 bp):

5'- ATA GCT TCA CAG CAG AGG TTA CAA CAC CAA TGC TGC TCC GCT ACC CGC GAT CTC ATG CAC CAG GGA GCG GCA AGA AGC CAG AGC TTC AAG ACA CCC CTA CCC CCG TGC AGG AGC TCA CAA AGA AAG TTC ACA GTG AGA TGG TTG GAT GTG TGT -3'

Surface probe, complementary to FwP tail (SP):

5'- TTC ATT GAG TTC GTC GTA AT 15T-$(CH_2)_6$-SH -3'

HRP-labelled probe complementary to RvP tail:

5'- HRP - ACT GGC CGT CGT TTT ACA. -3'

* - C3 spacer between the primer and the tail

DNA was dissolved in high purity deionized water (18 MΩ) produced with a Milli-Q RG system (Millipore Ibérica, Spain), aliquoted and diluted to the appropriate concentrations using Milli-Q water and used without further purification.

6.3.3. PEX protocol

The primer (0.7 μM) was mixed with template DNA (0.7 μM), natural dCTP, dTTP, dGTP and $dA^{NH2}TP$ (125 μM each) and a 1.25 U per sample of Klenow (exo-) polymerase. Reaction mixtures were incubated for 30 minutes at 37 °C. Amplification was verified via electrophoresis using 2.5 % agarose gel in 1 x Tris/Borate/EDTA buffer (TBE) for 30 min at 100 V.

6.3.4. PCR protocol

PCR was performed in a final volume of 50 µL volume using 1 x Master mix (Takara Shuzo Co., Ltd.), 800 nM primers, 25 µM natural dCTP, dTTP, dGTP, 200 µM $dA^{NH2}TP$, template DNA, Milli-Q water and KOD XL 2.5 U/µL DNA polymerase. For the non-specific control, Milli-Q water was used instead of specific DNA. PCR was performed using a Peltier Thermal Cycler (Bio Rad Laboratories S.A., Barcelona, Spain) with the following amplification program: initial denaturation at 95 ^{o}C for 3 minutes, followed by 24 cycles of denaturation at 95 ^{o}C for 30 seconds, annealing at 60 ^{o}C for 30 seconds and primer elongation at 72 ^{o}C for 30 seconds. Following the last cycle, primer elongation was continued for 5 minutes.

Successful amplification was verified via electrophoresis using 2.5 % w/v agarose gel in 1 x TBE for 30 min at 100 V. PCR dsDNA amplicons were purified according to the protocol provided with the Zymo Research DNA Clean & Concentrator kit. The purified amplicon concentration was determined using the SimpliNano microvolume spectrophotometer (Biochrom, Harvard Bioscience, Inc.) at λ=260 nm.

6.3.5. Recombinase Polymerase Amplification (RPA) protocol

RPA was performed as specified in the protocol provided by TwistDX. The reaction was carried out in a 50 µL volume using the customised dNTP-free formulation TwistAmp Basic kit, 240 nM PCR primers, DNA template, 15 mM magnesium acetate, 480 µM natural dCTP, dTTP, dGTP, 200 µM $dA^{NH2}TP$ and TwistAmp 1 x rehydration buffer. All reagents, with the exception of the template DNA and Mg-acetate were prepared in a master mix provided by TwistDx with a dried enzyme pellet,

which was aliquoted into 0.2 mL reaction tubes. Magnesium acetate was pipetted into the tube lids. Subsequently DNA was added into the tubes. The lids were closed and magnesium acetate centrifuged into the tubes to start the reaction. Further, the tubes were vigorously inverted 8-10 times to mix and again centrifuged. The RPA mixture was placed in an incubator block at 37 °C and incubated for 4 minutes, and then inverted 8-10 times to mix, and again centrifuged and placed back in the incubator block, for a further 20 minutes. The amplification was verified using 2.5 % w/v agarose gel via electrophoresis in 1 x TBE buffer at constant voltage 100 V for 30 minutes. RPA dsDNA amplicons were purified the same procedure as PCR products.

6.3.6. Direct hybridisation of PCR and RPA dsDNA products on maleimide microtitre plates by hybridisation via tail primers

Thiolated surface probe complementary to the tail of the amplicon was immobilised on the wells of a maleimide plate by addition of 100 µL of 200 nM solution in 10 mM PBS (pH 7.4), followed by an overnight incubation at room temperature and thorough washing with PBS containing 0.05% (v/v) Tween-20 (PBS-Tween). Blocking of any non-functionalised maleimide moieties was achieved via incubation with 200 µL of 6-Mercapto-1-hexanol in MilliQ for 1 h under shaking, and subsequent washing with PBS-Tween.

Hybridisation was performed by introducing 25 uL of amplicon dissolved in PBS to each of the wells. The reaction took place in an incubator with a fixed temperature of 37 °C for 1 hour. Following hybridisation, the plate was thoroughly washed with 200 µL of PBS-

Tween, 3-5 times. The amplicon was then hybridised with 50 µL of 10 nM HRP labelled probe complementary to the upper end tail for 30 minutes at room temperature under shaking conditions. After a final washing with PBS-Tween, the presence of the HRP-labelled reporter probe was observed via addition of 50 µL of TMB substrate for 5 minutes. The reaction was stopped by adding 50 µL of 1 M H_2SO_4. The absorbance was measured at 450 nm (SpectraMax 340PC384, bioNova científica s.l., Spain). In parallel, negative controls were included to demonstrate the selectivity of the assay: a) negative PCR/RPA control containing MilliQ instead of template DNA and b) non-specific surface probe.

To assess the sensitivity of the assay, amplification was carried out using different starting concentrations of *K. armiger* synthetic ssDNA (PCR: 0.4 nM, 0.04 nM, 0.004 nM, 0.0004 nM, 0.00004 nM, 0.01 nM, 0.000004 nM; RPA: 20 nM, 4 nM, 0.8 nM, 0.16 nM, 0.032 nM, 0.0065 nM).

6.3.7. Electrochemical detection

Glassy carbon electrodes (GCEs) were prepared as previously reported [25]. Briefly, GCEs were polished sequentially with 15, 3.0, 0.3, and 0.05 mm alumina for approximately 5 min, and were sonicated in EtOH and water for 5 min between each polishing step. GCE activation was performed in a saturated sodium carbonate solution for 5 min at a potential of 1.2 V versus Ag/AgCl to eliminate any hydrocarbon adsorbed from the environment; this was followed by sweeping of the potential for 40 cycles between 0 and 1.0 V versus Ag/AgCl at 250 mVs_1 in 0.1m sodium perchlorate.

To achieve hydrogenation of the GCE, it was immersed in 2M HCl and used as the cathode, and a platinum wire, immersed in 2M H_2SO_4

solution, was used as the anode. The solutions were separated by a glass tube with a porous membrane, and a potential of -5 V and 20 mA current were applied for 15 min. During the electrolytic process the solutions were stirred to prevent the accumulation of bubbles at the electrode surface.

Subsequetly, to achieve the electrochemical chlorination of hydrogenated GCE, the H-terminated GCE was used as an anode and immersed in a 3:1 ratio of 2M HCl/2M HNO_3. A platinum wire was used as the cathode and immersed in 2M H_2SO_4. Again, the solutions were separated by a glass tube with a porous membrane. A potential of +2 V and 20 mA current were applied for 5 min.

The immobilisation of DNA probes was achieved by pipetting a thiolated DNA surface probe (50 mL, 5 μm) in Trizma base (pH 8) with 6-MCH at 1:100 ratio onto the surface of a chlorinated GCE, followed by incubation for 24 h at room temperature. The electrodes were then rinsed with Tris-HCl buffer (pH 7.4) and dried under a stream of nitrogen. Subsequently, 25 μL of labelled DNA product (PEX, PCR, RPA) in Tris-HCl, pH 7.4 containing 1M NaCl, were hybridised with the surface tethered probe for 1 hour. Following washing, Square Wave Voltammetry (SWV) measurements were performed. The SWV parameters for oxidation of amino-labels (and guanine residues) were an initial potential of 0 V, a final potential of +1.5 V, a pulse amplitude of 25 mV, a frequency of 50 - 200 Hz and a potential step of 5 mV. The measurements were performed at ambient temperature in 0.1 M Tris-HCl, 0.2 M NaCl, pH 7.3 using an Autolab analyzer (EcoChemie, The Netherlands) in a three-electrode set-up (with the GCE as working, Ag/AgCl/1M KCl as reference and platinum wire as counter electrode). The voltammograms were baseline-corrected

by means of a moving average algorithm (Nova 2.1.2 software, EcoChemie).

6.4. Results and discussion

DNA was first amplified using conventional PEX (*Figure 6.3)* and PCR to test the efficiency of the incorporation of labelled dN^{X}TPs. *Figure 6.4* shows PCR amplicons obtained for synthetic DNA of *K. armiger*. No non-specific amplification was observed (*Fig. 6.4 lane N*). Subsequently, amplification was carried out using a modified RPA protocol, using 100 % substitution of dATP for the aminophenyl-labelled dATP and conventional PCR primers. RPA amplicons for synthetic DNA can be seen in *Figure 6.5 (lanes 1-7)*. No cross-reactivity was observed (*Fig. 6.5 lane N*). The obtained results thus demonstrate the possibility of RPA to amplify *K. armiger* with incorporation of labelled dN^{X}TPs.

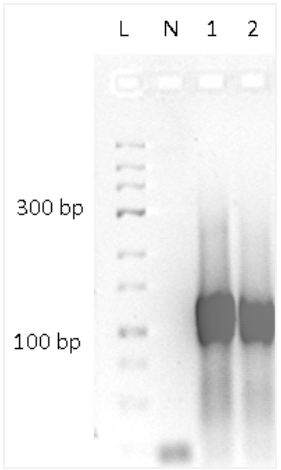


Figure 6.3 Electrophoresis of PEX amplicons in agarose gel 2.5 % of *Karlodinium armiger* DNA. **L** – 100 bp ladder; **1** – PEX product containing only natural dNTPs; **2** – PEX product containing dA^{NH2}TP; **N** - negative control.

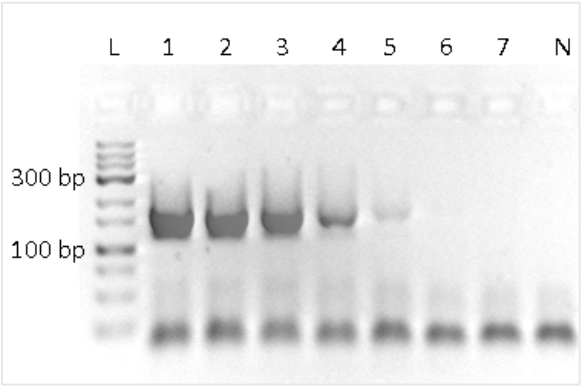


Figure 6.4 Electrophoresis of PCR amplicons in agarose gel 2.5 % of *Karlodinium armiger* DNA. **L** – 100 bp ladder; **1-7** – tail PCR amplicon containing $dA^{NH2}TP$ with different initial template concentration; **N** - negative control.

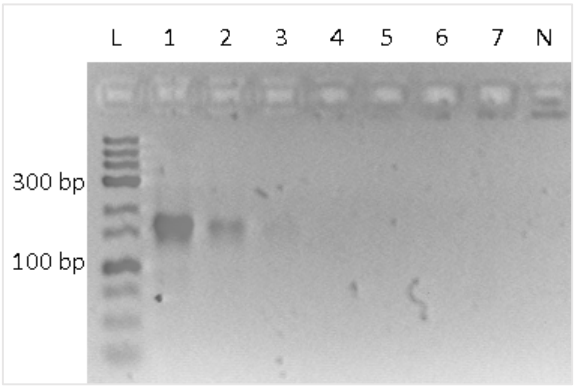


Figure 6.5 Electrophoresis of RPA amplicons in agarose gel 2.5 % of *Karlodinium armiger* DNA. **L** – 100 bp ladder; **1-7** – tail RPA amplicon containing $dA^{NH2}TP$ with different initial template concentration; **N** - negative control.

Once the incorporation of the labelled dATP and tailed primers in PCR and RPA amplification had been demonstrated using gel electrophoresis, the direct hybridisation of PCR and RPA dsDNA products on maleimide microtitre plates by hybridisation using the tailed primers was evaluated. As can be seen in *Figure 6.6*, PCR (A) and RPA (B) detection

of the specific target (dsDNA) was achieved by hybridisation of the amplicon to the surface-tethered probe and the HRP-labelled reported probe, complementary to the tail of the RvP. A selectivity study using a non-complementary surface probe clearly demonstrates the selectivity. *Fig. 6.6* shows the absorbance recorded for the calibration curve obtained using different initial ssDNA concentrations for the detection of *K. armiger* dsDNA. The linear range covers six orders of magnitude for PCR and four orders of magnitude for RPA. The limit of detection (LOD), obtained using PCR amplicons, was $3.45*10^{-15}$ M, whilst the LOD for the RPA amplicons was $2.11*10^{-11}$ M, determined as the concentration of the analyte at the mean blank signal plus three times the standard deviation of the blank.

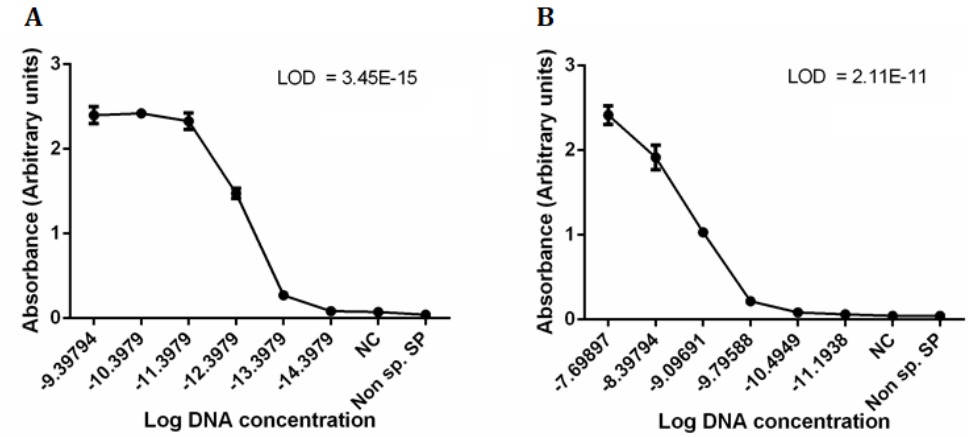


Figure 6.6 Absorbance of hybridisation assays for *Karlodinium armiger* on maleimide activated plates. **A** PCR calibration curve using different template concentration (n=3). NC - negative control using negative PCR control. Non-sp. SP – control using non-complementary surface probe. **B** RPA calibration curve using different template concentration (n=3). NC - negative control using negative RPA control. Non-sp. SP – control using non-complementary surface probe.

Electrochemical detection

Once direct hybridisation of amplified products had been demonstrated using the microtitre plate format, the system was successfully transferred to an electrochemical platform. Hybridisation and detection of DNA bearing the labelled nucleobases incorporated by PEX was analysed by SWV. The resulting voltammograms (*Figure 6.7*) revealed the presence of the amino labels due to a specific oxidation peak at around + 900 mV and a signal from the electrooxidation of guanine (G^{ox}). The achieved responses were compared with unlabelled PEX products to prove that the signal is not an artefact.

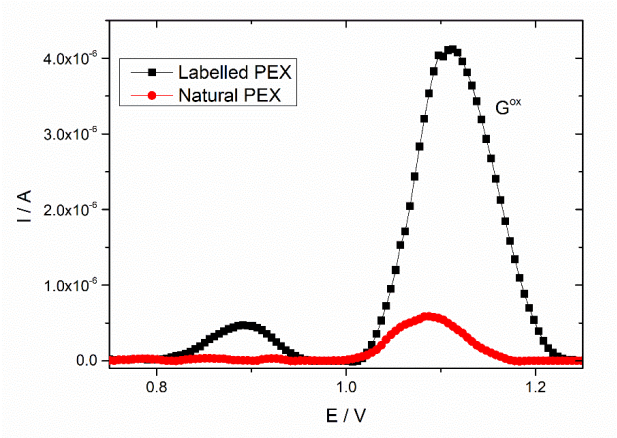


Figure 6.7 SWV responses of PEX products bearing aminophenyl labels (Labelled PEX) synthesised using mixtures of labelled $dA^{NH2}TPs$ with unlabelled PEX product (Natural PEX).

The results obtained from hybridisation of the PCR product on the surface of the GCE are shown in *Figure 6.8*. We see a clear response of aminophenyl-labelled PCR products as well as guanine electrooxidation. The natural non labelled PCR product showed only one peak corresponding to the guanine.

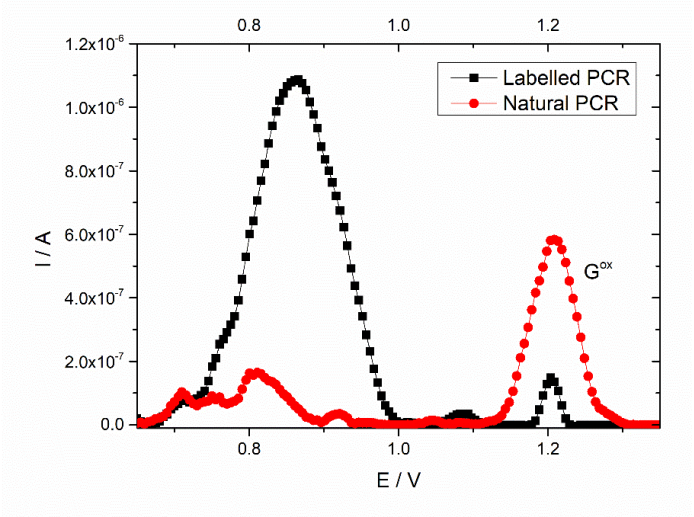


Figure 6.8 SWV responses of PCR products bearing aminophenyl labels (Labelled PCR) synthesised using mixtures of labelled $dA^{NH2}TPs$ with unlabelled PCR product (Natural PCR).

6.5. Conclusions

In conclusion, we have developed a facile optical and electrochemical detection system using aminophenyl-containing $dN^{X}TPs$ for the detection of *Karlodinium armiger*. The modified $dN^{X}TPs$ were efficiently incorporated by DNA polymerases in thermal (PCR) and isothermal (PEX and RPA) formats in order to form dA^{NH2}-modified amplicons. 100 % incorporation of modified $dN^{X}TPs$ using RPA was demonstrated for the first time. The chosen modification served as an excellent electrochemical label detectable by oxidation on GCEs. The tailed primer approach allowed simple hybridisation without the need of ssDNA generation.

6.6. Acknowledgements

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BIO2014-56024-C2-1). OM thanks the Generalitat de Catalunya for a FI pre-doctoral scholarship.

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CHAPTER 6

General conclusions

First of all we have demonstrated the success of the multiplex isothermal liquid-phase RPA concept for detection of several biological pathogens in "one-pot" reaction. Multiplex analysis of different nucleic acid targets simultaneously has a great value in molecular diagnostics as biological agents require urgent attention and development of an easy, rapid, affordable and reliable system for their detection

Further we have demonstrated the success of the isothermal solid-phase RPA concept as a DNA amplification method combined with optical detection system. We proved the ability of RPA to use both ssDNA and dsDNA as a template for amplification, achieving the LOD of $4.04*10^{-13}$ and $3.14*10^{-16}$ M, respectively. The system demonstrated high specificity with negligible responses to non-specific targets. Moreover, solid-phase RPA approach overcomes not only the limitations present in regular PCR amplification techniques, but also many of the regular RPA-based published methods, due to the simplicity of the system.

The general objective of this doctoral thesis was to develop isothermal amplification protocols compatible with a portable, easy-to use device that can be deployed for analysis of genetic material at the point-of-care/need. So next the solid-phase concept has been applied for the electrochemical detection. Further optimisation allowed us to improve the analytical parameters of the platform in terms of LOD, signal-to-noise ratio and steps required.

The combination of isothermal RPA with redox labelled dNTPs for the amplification and detection of toxic microalgae was an innovative method that permitted incorporation of labels throughout the

amplification process facilitating direct electrochemical detection of the RPA products and an inherent shortening of assay time. The presence of redox labels through the whole length of amplicon facilitated significant signal enhancement, allowing easier detection of the chosen targets. Moreover, this approach brings us closer to an ASSURED "sample-in response-out" system that should be as simple as possible with a minimum number of steps and reagents. Detection of the amplified material has been carried out by hybridisation with labelled primers, incorporation of labelled amynophenyl-modified dNTPs during the amplification and electrochemical detection.

The main added value of this thesis is that it presents a flexible solution for detecting DNA with biosensors, exploiting a general concept of liquid-phase and solid-phase isothermal amplification and detection, thus integrating two nucleic acid tests, PCR and microarrays, in one single device.

Annexes

UNIVERSITAT ROVIRA I VIRGILI
DEVELOPMENT OF DIAGNOSTIC PLATFORM FOR DETECTION OF BIOLOGICAL AGENTS AND TOXIC MICROALGAE USING ISOTHERMAL AMPLIFICATION.
Olena Mayboroda

Annex 1 Currently developed RPA assays

Target RNA	Template	Biological sample	Performance (time and LOD)	Amplification tº	Detection method	Multiplexing	Ref.
Avian influenza H5N1 HA gene	RNA	Tracheal swabs from field cases	1 copy in 20 min	42 º	Real time fluorescence	No	1
Bovine coronavirus	RNA	Fecal and nasal scabs	19 molecules in 10-20 min	42 º	Real time fluorescence	No	2
Cancer fusion genes	RNA	Urine	1000 copies in 15 min	37 º	Real time fluorescence	Yes	3
Chikungunya Virus	RNA	Cell culture	80 genome copies in 15 min	42 º	Real time fluorescence	No	4
Coxsackievirus A6	RNA	Stool specimens	202 copies in 20 min	40 º	Real time fluorescence	No	5
Dengue Virus	RNA	Serum samples	10 copies in <20 min	40 º	Real time fluorescence	No	6
Dengue Virus	RNA	Plasma samples	10-100 copies in 3-7 min	38 º	Real time fluorescence	No	7
Ebola virus	RNA	Spiked plasma dilutions	21 copies in 7 min	42 º	Real time fluorescence	No	8
Ebola virus	RNA	Serum, swab	5 genome copies in 15 min	42 º	Real time fluorescence	No	9

Ebola Virus	RNA	Blood, swabs	10 copies in 15 min	42 º	Real time fluorescence	No	[10]
Ebola virus	RNA	Clinical samples	10^7 copies/µL in 20 min	40 º	Real time fluorescence Paper microfluidics device	Yes	[11]
Epidemic Human Noroviruses	RNA	Stool	3.40 ± 0.20 $\log_{10}$ genomic copies (LGC) in 30 min	40 º	Real time fluorescence	No	[12]
Foot and mouth disease virus	RNA	Vesicular material, saliva, serum, blood, swabs	1436 copies in 4-10 minutes	42 º	Real time fluorescence	No	[13]
Hepatitis B, C HIV	Viral DNA/RNA	Whole blood	1000 viral genetic copies per millilitre in 10-30 min	37 º	Quantum dot barcode smartphone optical device	Yes	[14]
Hop stunt viroid	RNA	Leaf tissues	10 ng/µL in 20 min	39 º	Lateral flow	No	[15]
Influenza A (H7N9) virus	RNA	RNA standards	10-100 copies in 2-7 minutes	42 º	Real time fluorescence	No	[16]
Little cherry virus 2	RNA	Budwood or leaf cherry tissue	15 min	39 º	Lateral flow	No	[17]

Marburg virus	RNA	Spiked plasma dilutions	21 copies in 8 min	42 º	Real time fluorescence	No	8
Middle East respiratory syndrome coronavirus	RNA	Samples collected from MERS-CoV-infected patients	10 copies in 10 min	42 º	Real time fluorescence	No	18
miRNA	RNA	Urine	40 copies/pg in 15 min	37 º	Real time fluorescence	Yes	19
Peste des petits ruminants virus	RNA	Spiked tissues	100 copies in 7 min 150 copies in 25 min	40 º 39 º	Real time fluorescence Lateral flow	No	20
Plum pox virus	RNA	Crude plant extracts of peaches, apricot, cherry and European plum	1 fg in 20 min	39 º	Real time fluorescence Lateral flow	No	21
Porcine reproductive and respiratory syndrome virus	RNA	Tissue, serum	70 copies in 20 min	40 º	Real time fluorescence	No	22
Prostate cancer cells	RNA	Urine	100 copies in 15 min	41 º	SERS	Yes	23

Prostate cancer cells	RNA	Cell culture, urine and tumor samples	100 copies in 20 min	41 º	SERS	Yes	24
Pseudomonas syringae, F.oxysporum, Botrytis cinérea, F. oxysporum cubense, Cucumber mosaic virus, Bovine Herpesvirus 1, E. coli, HIV, P. Falciparum, Mycobacteria Tuberculosis	DNA/RNA	Stems, leaves, cells, blood cultures, water	N/A in 15 min	37 º	Agarose gel Naked eye	Yes	25
Rift Valley fever virus	RNA	RNA extracted from cells	10-19 copies in 8-20 min	42 º	Real time fluorescence	No	26
Rift Valley fever virus	RNA	Spiked plasma dilutions	19 copies in 7 min	42 º	Real time fluorescence	No	8
Rose rosette virus	RNA	Leaf, stem, petal	1 fg/ul in 20 min	42 º	Agarose gel Real time fluorescence	No	27
Rose rosette virus	RNA	Rose plants	1 fg/µl in 20 min	42 º	Real time fluorescence	No	28

Schmallenberg Virus and Bovine Viral Diarrhea Virus	RNA	Serum and blood samples	5 * 10^4 copies in 20 min	42 º	Real time fluorescence	No	29
Sigma virus	RNA	Spiked plasma dilutions	16 copies in 4 min	42 º	Real time fluorescence	No	8
Sudan virus	RNA	Spiked plasma dilutions	17 copies in 8 min	42 º	Real time fluorescence	No	8
TMPRSS2-ERG Gene in Prostate Cancer cells	RNA	Urine	10^3 – 10^5 in 20 mni	43 º	Colorimetry Electrochemistry	No	30
Tomato chlorotic dwarf viroid	RNA	Samples of leaves of viroid-infected and mock inoculated tomato or petunia plants	100 fg-1 pg in 15 min	39 º	Lateral flow	No	31
Type 2 porcine reproductive and respiratory syndrome virus	RNA	Lymph node, lung, spleen and liver	690 copies in 20 min	40 º	Real time fluorescence	No	32
Yam mosaic virus	RNA	Leaf samples	14 pg in <30 min	37 º	Real time fluorescence	No	33

Yellow Fever Virus	RNA	Cell culture and mosquitoes	<21 genome equivalent copies per reaction in <20 min	39 º	Real time fluorescence Lateral flow	No	34
Zika Virus	RNA	Urine	100 copies in 15 min	40 º	Real time fluorescence	No	35

Target DNA	Template	Biological sample	Performance (time and LOD)	Amplification tº	Detection method	Multiplexing	Ref.
Bacillus anthracis	DNA	Backbone plasmid standards	16 copies in 8 min	42 º	Real time fluorescence	No	8
Bacillus subtilis SpoB	DNA	Cell culture	100 copies in 30 min	37 º	Real time fluorescence	No	36,37
Banana bunchy top virus	ssDNA	Asymptomatic banana leaf samples	10^{-5} dilution in 30 min	37 º	Agarose gel	No	38
Begomoviruses	ssDNA	Tomato, tobacco and bean plants	9.6 pg in 30 min	37 º	Agarose gel	No	39
Borrelia burgdorferi	DNA	Patient serum, cell lines	25 copies in 20 min	37 º	Lateral flow	No	40
Botrytis cinerea, Pseudomonas syringae, Fusarium oxysporum	DNA	Infected leaves	1.9 fmol in 20 min	37 º	SERS	Yes	41
Brucella	DNA	Blood samples	3 copies in 20 min	38 º	Real time fluorescence	No	42
Campylobacter coli and Campylobacter jejuni	DNA	Eggs, chicken meat, chicken broth	1-10^3 CFU/ml in 20 min	45 º	Real time fluorescence	Yes	43

Candidatus Phytoplasma oryzae	DNA	Leaf and stem of Napier grass	10 organisms in 20 min	39 º	Real time fluorescence Lateral flow	No	44
Canine parvovirus type 2	ssDNA	Fecal samples	10 copies in 20 min	38 º	Agarose gel	No	45
Caprine arthritis-encephalitis virus	DNA	Blood samples	10 copies in 30 min	37 º	Lateral flow	No	46
Chlamydia trachomatis	DNA	First-void urine samples	100 copies in 10 min	38 º	Lateral flow	No	47
Chlamydia trachomatis	DNA	Synthetic DNA	$1*10^5$ copies in 10 min	44 º	Optical detection	No	48
Closely-related species with naturally occurring mismatches	DNA	Genomic DNA from bacterial species	N/A in 20 min	39 º	Agarose gel	No	49
Common safety threats in foodstuffs	DNA	Reference strains	1.3–5.3 μg g^{-1} for ingredients and 6–13 CFU mL^{-1} for pathogen cultures in 40 min	40 º	Microtiter plate	No	50
Cryptosporidiosis	DNA	Infected stool samples	1–10 oocysts in 30 min	37 º	Agarose gel Lateral flow	No	51
Cryptosporidium spp. oocysts	DNA	Dairy cattle feces	0.5 oocysts in 25 min	37 º	Lateral flow	No	52

Cytochrome-c	ssDNA	HepG2 cell line	10 ng/mL in 15 min	37 º	Real time fluorescence	No	53
E. coli or K. pneumoniae	DNA	Human clinical sample	< 10 copies in 30 min	39 º	Thin Film Transistor Nanoribbon	No	54
EGFR Mutation	DNA	Cell lines	20 pg in 5 min	37 º	Naked eye	No	55
Escherichia coli	DNA	Cell culture	1 copy in 15 min	39 º	Microfluidic device	No	56
Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Enterococcus faecalis	DNA	Urine	100 copies in 20 min	42 º	Real time fluorescence	No	57
ESKAPE Bacterial Pathogens	DNA	Cell culture	10 copies in 30 min	37 º	Microfluidic platform	Yes (parallelised)	58
Fasciola hepatica	DNA	Stool	1 pg/µL in 30 min	38 º	Lateral flow	No	59
Feline Herpesvirus-1	DNA	Nasal and ocular conjunctival swabs	10^2 copies in 20 min	39 º	Real time fluorescence	No	60
Francisella tularensis	DNA	Backbone plasmid standards	19 copies in 10 min	42 º	Real time fluorescence	No	8

Francisella tularensis	DNA	Genomic DNA	$2*10^5$ - $4*10^6$ copies in 40-60 min	37 º	Microtiter plate Chronoamperometry	No	61
Francisella tularensis	DNA	Synthetic DNA	$6*10^5$ copies in 60 min	37 º	Ring resonator	No	62
Francisella tularensis	DNA	Hare	19.01 molecules in 20 min	42 º	Real time fluorescence	No	63
Francisella tularensis	DNA	Spleen and liver tissue from hares	$1*10^{-15}$ M in 90 min	37 º	Electrochemical detection	No	64
Genetically Modified Crops	DNA	Maize, rice, cotton, and soybean	100 copies in 15-20 min	39 º	Real time fluorescence	No	65
Genetically modified organisms (GMOs)	DNA	Food samples	7 μg g^{-1} in 40 min	37 º	Digital versatile discs (DVD)	Yes (parallelised)	66
Genotyping	DNA	Buccal smear samples	10^3 pg in 40 min	37 º	Colorimetry	Yes (parallelised)	67
Genuity Roundup Ready 2 Yield (RR2Y) material in soybean (Glycine max)	DNA	Seed Extracts	10 copies in 15-20 min	39 º	Lateral flow	Yes	68
Giardia duodenalis	DNA	Stool	50 cysts in 30 min	37 º	Lateral flow	No	69

Giardia, Cryptosporidium, and Entamoeba	DNA	Spiked Stool	368 – 425 copies in 35 min	37 º	Lateral flow	Yes	[70]
Group B streptococcus	DNA	Vaginal swabs	6-12 copies in 20 min	40 º	Real time fluorescence	No	[71]
Group B Streptococci	DNA	Vaginal, anal samples	20 copies in 30 min	39 º	Real time fluorescence	Yes	[72]
GTS 40-3-2 soybean	DNA	Seeds of soybean	100 copies in 20 min	37 º	Naked eye	No	[73]
HIV	DNA	Plasmid containing the HIV gag gene	10 copies in 15 min	37 º	Lateral flow	No	[74]
HIV-1	DNA	Human genomic DNA	10 copies in 20 min	Body temperature	Lateral flow	No	[75]
HIV-1	DNA	ACH-2 cell line	3 copies in 20 min	39 º	Real time fluorescence Lateral flow	No	[76]
HIV-1	DNA	ACH-2 cell line	10 copies in 20 min	10 – 44 º	Agarose gel Lateral flow	No	[77]
HIV-1	DNA	Plasmid	10^3 copies in 30 min	37 º	Lateral flow	No	[78]
HIV-1	DNA	ACH-2 cell line	>10 copies in 20 min	39 º	Lateral flow	No	[79]
HIV-1	DNA	DNA from oocysts	200 copies in 20 min	39 º	Real time fluorescence	No	[80]
HRAS (Harvey RAS) gene	DNA	T24 human bladder	500 fg μl^{-1} in 20-30 min	37 º	Silicon microring resonator during	No	[81]

		cancer cell line					
Human adenovirus 41, Phi X 174, Enterococcus faecalis	DNA	Plasmid standards	35 GU/µL, 1 GU/µL, 5×10^3 GU/µL in 40 min	39 º	Microarray chip	Yes	[82]
Human cancer cell lines	DNA	Cells and blood	0.5 ng in 30 min	37 º	Naked eye	No	[83]
Human Papillomavirus Type 16 and Type 18	DNA	Clinical samples	100 copies in 20 min	37 º	Agarose gel Real time fluorescence	No	[84]
Hypodermal and hematopoietic necrosis virus (IHHNV)	ssDNA	Shrimp, Chinese mitten crab	4 copies in 20 min	39 º	Real time fluorescence	No	[85]
Klebsiella pneumoniae	DNA	Human urine	1000 bacteria/ml in 15 min	39 º	Microfluidic chip	No	[86]
Klebsiella pneumoniae carbapenemase New Delhi metallo-β-lactamase	DNA	Cell culture	10 copies in 25 min	39 º	Digital Microfluidic Device Real time fluorescence	Yes	[87]
Leishmania donovani	DNA	Buffy coat, skin biopsy	39 molecules in 15 min	42 º	Real time fluorescence	No	[88]

Leishmania infantum	DNA	Dog blood	0.8 parasites per mL of blood in 10 min	37 º	Chronoamperometry	Yes	89
Leishmania Viannia spp.	DNA	Reference or clinical strains	0.1 parasite in 20 min	45 º	Lateral flow	No	90
Leptospira	DNA	Serum, blood	≤10 fg in 25 min	38 º	Real time fluorescence	No	91
Listeria monocytogenes	DNA	Pork, chicken, beef, fish, and UHT milk	1360 CFU/ mL in 20 min	37 º	Lateral flow dipstick	No	92
Listeria monocytogenes	DNA	Genomic DNA	$4*10^{-3}$ copies in 30 min	39 º	Picoliter Well Array Chip	No	93
Listeria monocytogenes	DNA	DNA concentration standards	N/A in 30 min	39 º	Microfluidic disks	No	94
Listeria monocytogenes	DNA	Spiked whole blood	$2*10^{4}$ cells/ml in 25 min	40 º	Real time fluorescence	No	95
Lumpy skin disease virus	DNA	Skin nodules	179 molecules in 15 min	42 º	Real time fluorescence	No	96
Madurella mycetomatis	DNA	Surgical biopsy specimens	0.47 ng in 40 min	39-40 º	Agarose gel	No	97
Mangalica meat	DNA	Pig, cattle, chicken and turkey	1 copy in 30 min	39 º	Lateral flow	No	98

		muscle, wild boar and deer samples, sausage and liver paté					
Methicillin resistant S. Aureus (MRSA)	DNA	Genomic DNA	10 copies in 30 min	37 º	Agarose gel Lateral flow	Yes	36
MRSA	DNA	Genomic DNA	300 copies/mL in 30 min	39 º	SlipChip	No	99
MRSA	DNA	Human whole blood samples	10^3 copies in 30 min	37 º	Microfluidic chips	No	100
MRSA	DNA	Nasal and groin swabs	N/A in 20 min	39 º	Real time fluorescence	Yes	101
MRSA	DNA	N/A	<10 copies in <20 min	37 º	Microfluidic unit	Yes (parallelised)	102
Mycobacterium avium subsp. paratuberculosis	DNA	MAP positive blood, sperm, faecal and tissue	500 fg in 15 min	42 º	Real time fluorescence	No	103
Mycobacterium tuberculosis	DNA	Sputum, respiratory washes (bronchial and tracheal)	6.25-20 fg in <20 min	39 º	Real time fluorescence	No	104
Mycobacterium tuberculosis	DNA	Cell cultures	1 CFU in 20 min	38 º	Electrochemical Assay	No	105

Mycobacterium tuberculosis	DNA	Cell culture	1-10 CFU in 20 min	38 º	Naked eye colorimetry Electrochemistry	No	106
Mycobacterium tuberculosis	DNA	Patient sputum samples	26 pg/mm^2 in 20 min	37 º	Silicon biophotonic sensor	No	107
Mycoplasma capricolum subsp. capripneumoniae	DNA	Pleural fluid samples, lung tissue specimens	5-500 CCU/reaction in 15 min	42 º	Real time fluorescence	No	108
Neisseria gonorrhoeae, Salmonella enterica, MRSA	DNA	Genomic DNA	10-100 copies in 40 min	38 º	Microarray scanner	Yes	109
Orf virus	DNA	Skin, lymphatic nodes, liver, lungs, stomach and kidney, nasal swabs	80 copies in 25 min	37 º	Lateral flow	No	110
Orf virus	DNA	Skin, lymphatic nodes liver, lungs, stomach and kidney	100 copies in 20 min	37-39 º	Real time fluorescence	No	111

Orientia tsutsugamushi Rickettsia typhi	DNA	Patient blood, infected mice, spiked normal human plasma	20 – 40 copies in 20 min	37 - 39 º	Real time fluorescence Lateral flow	No	112
Peanut, GMO, Salmonella spp., Campylobacter spp.	DNA	Reference strains, certified reference materials	48.7-900 ng in 45 min	37 º	Digital versatile discs (DVD)	Yes (parallelised)	113
Penaeus stylirostris densovirus	DNA	Shrimps	100 pg in 30 min	37 º	Agarose gel Real time fluorescence Lateral flow	No	114,115
Phytophthora infestans	DNA	Potato leaves	50 fg/µl in 30 min	39 º	Real time fluorescence	No	116
Phytophthora sojae, Phytophthora sansomeana	DNA	Soybean seedlings	10 pg in 25 min	39 º	Real time fluorescence	No	117
Piscirickettsia salmonis	DNA	Genomic DNA	$5*10^{-8}$ µg/ml in 40 min	37 º	Chronoamperometry	No	118
Plasmodium	DNA	Plasmid sequences	50 copies in 30 min	37 º	Lateral flow	No	119
Plasmodium	DNA	Spiked human whole blood	<1 parasite μL^{-1} in 30 min	37 º	Mach-Zehnder Interferometer sensor	No	120

Plasmodium falciparum	DNA	Genomic DNA	100 fg in 10 min	38 º	Lateral flow	No	121
Porcine circovirus 2	ssDNA	Lymph node, liver, lung, spleen and serum	103 copies in 20 min	38 º	Real time fluorescence	No	122
Porcine parvovirus	DNA	Lymph node, lung, spleen, kidney and duodenum	103 copies in 20 min	39 º	Real time fluorescence	No	123
Porcine parvovirus (PPV)	ssDNA	Serum, liver, kidney, lymph node, spleen and duodenum	300 copies in 9 min 400 copies in 20 min	38 º	Real time fluorescence Lateral flow	No	124
Prostate cancer biomarker	DNA	Urine	50 ng in 30 min	37 º	Naked eye colorimetric detection	No	125
Pseudomonas syringae	DNA	Infected leaves	15 copies in 20 min	37 º	Agarose gel Differential pulse voltametry	No	126
Pseudorabies virus	DNA	Heart, spleen, tonsil, lung and lymph nodes	100 copies and 160 copies in 20 min	39 º	Real time fluorescence Lateral flow	No	127
S. mansoni, HIV-1 clade B, S. hemato- bium, P.	DNA/RNA	Mice serum, spiked human serum and	0.5 fg in 20 min RPA +	37 º (RPA) 60-65 º (LAMP)	Colorimetric or fluorescent dye and smartphone	Yes	128

falciparum, Schistosoma japonicum, Brugia ma- layi, Strongyloides stercoralis, drug-resistant Salmonella, Zika virus (ZIKV)-America strain (mex 2–81, Mexico), ZIKV-Africa strain (MR 766, Uganda), human papil-loma virus (HPV)-58, HPV-52, HPV-35, HPV-45, HPV-18, and HPV-16		whole blood, spiked urine samples	15-20 min LAMP				
Salmonella	DNA	Food and clinical samples	6-30 CFU/ml in 40 min	37 º	Digital versatile discs (DVD)	Yes (parallelised)	129
Salmonella bacteria	DNA	Milk, chicken breast, egg samples	20 fg in 20 min	39 º	Lateral flow	No	130

Salmonella enterica	DNA	Eggs and chicken meat	10-100 CFU/g in 10 min	37 º	Real time fluorescence	No	131
Salmonella enterica, Escherichia coli O157:H7, Vibrio parahaemolyticus	DNA	Milk samples	4 cells in 30 min	39 º	Centrifugal microdevice	Yes (parallelised)	132
Schistosoma japonicum	DNA	Fecal samples	5 fg in 15-20 min	39 º	Lateral flow dipstick	No	133
Schistosoma haematobium	DNA	Spiked urine	100 fg in 20 min	37 º	Lateral flow	No	134
Schistosoma japonicum	DNA	Stool and serum samples	0.9 fg in 15 min	39 º	Real time fluorescence	No	135
Shrimp White Spot Syndrome Virus	DNA	Shrimp	5 copies in 7 min	39 º	Real time fluorescence	No	136
Streptococcus pneumoniae	DNA	Whole blood	<1 CFU in 20 min	40 º	Real time fluorescence	No	137
Synthetic target	DNA	Synthetic DNA	190 amol in 15 min	37 º	Lateral flow	No	138
Theileria annulata	DNA	Blood samples	2 pg in 20 min	37 º	Lateral flow	No	139
Toxin B gene of Clostridium difficile	DNA	Cell culture	1 fg in <20 min	39 º	SlipChip platform	No	140

Toxoplasma gondii	DNA	Soil and water samples	0.1 oocyst in 20 min	37 º	Lateral flow	No	141
Transgenic Rice Kefeng 6	DNA	Rice	500 copies in 10-20 min	39 º	Real time fluorescence	No	142
Variola virus	DNA	Backbone plasmid standards	16 copies in 10 min	42 º	Real time fluorescence	No	8
Vibrio cholerae	DNA	Aquacultured shrimps, clams, and fishes	5 copies in 20 min	39 º	Real time fluorescence	No	143
Vibrio owensii	DNA	Plasmid	2 copies in 9 min	39 º	Real time fluorescence	No	144
Yersinia pestis	DNA	Backbone plasmid standards	16 copies in 8 min	42 º	Real time fluorescence	No	8
Yersinia pestis	ssDNA/ dsDNA	Synthetic and genomic DNA	$3*10^{-16}$ M in 30 min	37 º	Microtiter plate	No	145
β-Conglutin	DNA	Lupinus albus	$3.5*10^{-11}$ M in 25 min	37 º	Microtitre plate	No	146
β-Conglutin	ssDNA	Synthetic DNA	9 fM in 15 min	RT	Lateral flow	No	147

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